# THE ROLE OF SEX PHEROMONES IN THE REPRODUCTIVE ISOLATION OF *HELIOTHIS* SPECIES (LEPIDOPTERA: NOCTUIDAE)

BY

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For Margaret Louise Ferguson Teal and Barbara Allison Teal with all my love.

PEAT

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# THE ROLE OF SEX PHEROMONES IN THE REPRODUCTIVE ISOLATION OF *HELIOTHIS* SPECIES (LEPIDOPTERA: NOCTUIDAE)

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Chemical, behavioral, morphological, and physiological aspects of the sex pheromone communications systems of *Heliothis virescens* (F.) and *H. subflexa* (Gn.) were studied under laboratory and field conditions.

Behavioral analyses of the semiochemically induced inter- and intra-specific reproductive interactions between *H. virescens* and *H. subflexa* were conducted in the laboratory. The reproductive behavior of *H. virescens* was broadly categorized into 2 distinct phases dependent upon the interactive forces governing each. Precourtship behaviors include female calling, male activation, and orientation behaviors, all of which rely on a unidirectional flow of behavioral information and are rather stereotyped in their sequence. Courtship involves numerous variably committed interactions between the sexes. Nonetheless, a common courtship sequence is apparent, with several points at which reproductive isolation from closely related species could be effected. *H. subflexa* males were incapable of orienting toward calling *H. virescens* females, indicating that semiochemically induced reproductive isolation acts at a distance. *H. virescens* males were

capable of orienting toward calling H. subflexa females but the majority of males either failed to enter into or complete courtship. Therefore, close range reproductive isolation appears to maintain the genetic integrity of this pair. The pheromone blend extracted from H. subflexa female glands did not induce H. virescens males to enter taxis, indicating that there are differences between the pheromone blend released by calling females and that extracted from the glands.

The ovipositor of H. virescens was found to have 2 areas of glandular tissue. The 1st area, located in the intersegmental membrane between abdominal segments 8 and 9 + 10, has the form of a ventrolateral chevron. The 2nd glandular area, situated in the dorsal valves (papillae annales), is composed of an area of columnar epidermal cells, similar to cells in the intersegmental membrane, and of trichogenous glands associated with tubular setae. Extracts from these 2 glandular areas were assayed for their ability to elicit both EAG responses and male sexual behavior in bioassays. EAG studies indicated that extracts from both glandular sites were perceived by the male antennae, and wind tunnel bioassays confirmed the role of extracts from each glandular site in the elicitation of male sexual behaviors. However, due to disparate response levels to individual and combined extracts, it is hypothesized that each glandular site maintains a different pheromone blend and the admixture of the 2 sites is necessary for maximizing male sexual behaviors.

Gas chromatographic and mass spectral analysis of extracts obtained from the ovipositor of calling H. subflexa females revealed

the presence of 8 compounds which were identified as hexadecanal, (Z)-9-hexadecenal, (Z)-11-hexadecenal, (Z)-7-hexadecen-1-ol acetate, (Z)-9-hexadecen-1-ol acetate, (Z)-11-hexadecen-1-ol acetate, (Z)-9-hexadecen-1-ol, and (Z)-11-hexadecen-1-ol. Although the whole blend was found to be an effective male attractant, the deletion of the alcohols from the blend increased trap captures considerably. Further, although the binary mixture of (Z)-9-hexadecenal and (Z)-11-hexadecenal caught some male H. subflexa, significant increases in captures were noted when the 3 acetate components were included in the blend.

# CHAPTER I
# LITERATURE REVIEW AND RESEARCH AIMS

## Systematics

The subfamily Heliothidinae (Lepidoptera: Noctuidae) is composed of an assemblage of 14 genera including some 158 species (Hardwick, 1970). Until recently the major North American pests of this subfamily were considered members of a single genus, Heliothis, which included Heliothis armigera (Hbn) and H. virescens (F.) (Brazzel et al., 1953). The North American form of H. armigera was subsequently given species status (Common, 1953) and renamed Heliothis zea (Boddie) by Todd (1955). Through further reclassification (see Hardwick, 1965) the original species, H. armigera, was divided into 6 species groups and reclassified in the genus Helicoverpa (Hardwick, 1965). The basis of Hardwick's classification lies in the presence of cornutii on the male vesica and the female appendex bursae which is coiled or alternatively dilated and constricted. The genus Heliothis is, according to Hardwick (1970), distinguished from Helicoverpa in that male Heliothis have a denticulate bar at the base of the vesica adjacent to the right margin of the aedeagus and females have an appendex bursa of approximately the same size as the fundus bursae. To avoid the taxonomic arguments surrounding this species, Helicoverpa (Heliothis) zea is herein referred to as Heliothis zea.

Based on Hardwick's monograph (1970) the genus Heliothis is composed of 13 species including H. virescens (F.) and H. subflexa

(Gn). The latter species is so morphologically similar to H. virescens that its species status was under considerable question until McElvare (1941) suggested the restoration of H. subflexa to the specific rank on the basis of an absence of wing banding and internal genitalical differences. Brazzel et al. (1953) negated the use of wing banding, noting that only male H. subflexa had immaculate wings. Their studies on genitalic structure were, however, in general agreement with those of McElvare (1941). Similarly Furr et al. (1974) have concluded that male genitalic differences were sufficient to segregate both species and H. virescens male X H. subflexa female hybrids.

The eggs and first 2 larval instars of both species are identical; however, in the 3rd instar H. virescens diverges, developing spicuoles on setigerous tubercles on abdominal segments 1, 2, and 8, and a basal process on the oral face of the mandible which becomes tooth-like in later instars (Brazzel et al., 1953). Larval H. subflexa are distinguished by their habit of feeding solely on ground cherry, Physalis spp., and development of short microspines on the basal half of the abdominal tubercles (Brazzel et al., 1953). The pupae of both species are apparently indistinguishable.

## Seasonal Distributions

Due to the polyphagous nature of both H. zea and H. virescens range limitations are apparently solely dependent upon seasonal climatic conditions, with northward extensions governed by the maximum distances travelled by individuals of successive summer generations (Hardwick, 1965). Hardwick states that few, if any, individuals are capable of

overwintering as pupae above 40°N latitude; hence pest outbreaks in these northern areas (see Beirne, 1971) are the result of seasonal influxes. The number of generations per year increases from north to south, there being 4-5 generations in Louisiana, Arkansas, and North Florida (Brazzel et al., 1953; Snow and Brazzel, 1965), with the penultimate generation overwintering in pupal diapause while the last is generally killed by the first hard frost (Gentry et al., 1971). In subtropical areas there is no cold-induced diapause and hence a continuum of generations per year (see Hardwick, 1965). In addition to the effects of climate on range limitations, H. subflexa is further limited by the distribution of its host plant, the ground cherry. Although little is known about the population dynamics of this species it probably closely approximates that of H. virescens (see Brazzel et al., 1953).

Life Cycles

Exceptionally detailed descriptions of the life histories of H. virescens and H. subflexa have been given by Brazzel et al. (1953) and that of H. zea by Hardwick (1965). As all 3 have quite similar life cycles, the following is a generalized synopsis of the observations of the above authors.

Eggs are laid over an extended period, between dusk and dawn, although late in the season at higher latitudes adults can be found ovipositing in the late afternoon and evening. Oviposition occurs on or near flowers, with terminal growth being the preferred site. Larvae emerge from their eggs after an incubation period of 3-14 days, depending upon climatic conditions, and proceed to move through the

terminal buds to feed from the undersides of leaves. Larvae of middle stadia then move to the reproductive parts of the plant which not only provide protection from predators but also from pesticides during the final phases of larval development. Both the number of larval instars (5-7) and larval feeding period are variable and dependent upon temperature and the nutritive value of the food consumed. Larval development may be completed in as little as 2 weeks at 27-30°C when feeding on cotton bolls, or as prolonged as a month on tomato or tobacco leaf buds at temperatures in the low 20°C's. In response to food having low protein content, such as alfalfa, larvae of mid and later instars may become carnivorous, feeding on the pupae of other Lepidoptera, or cannabilistic when in high numbers.

After completion of feeding, larvae enter a prepupal stage of 2-3 days during which time they migrate down the host plant, burrow into the soil, and construct a pupal cell at 3-23 cm beneath the surface. Pupal periods are variable and again dependent upon climatic factors. In northern and arid climates pupae enter an arrested state of development during inclement months while during the growing season and in southern temperature climes the pupal period is generally 12-33 days.

Reports on adult longevity are somewhat ambiguous; mean values of 5.5 and 8.4 days are reported for unmated and mated females by Callahan (1958a), while values reported by both Hardwick and Brazzel et al. are at least twice that length. The longer figures seem more reasonable in view of the long northward migrations, with pest outbreaks of both H. zea and H. virescens being reported as far north as central Saskatchewan

(Beirne, 1971), and estimated northward migration of 440 km by at least some individuals of each generation (see Hardwick, 1965).

Host Plants and Economic Damage

Although both H. zea and H. virescens show considerable preference for plants of the families Leguminosae and Solanaceae, the extensive host lists of wild and cultivated plants, including both graminaceous and malvaceous plants, indicate that they are polyphagous (Barber, 1937; Brazzel et al., 1953; Snow and Brazzel, 1965; Green and Thurston, 1971). Reports also indicate that wild hosts, principally wild geranium and winter legumes, support the first larval generations with subsequent generations developing on cultivated crops (Brazzel et al., 1953; Snow and Brazzel, 1965).

Losses resulting from damage to cultivated crops in the United States by H. zea alone were estimated in the hundreds of millions of dollars by Hardwick (1965). If H. virescens is included and one considers the high levels of pesticide resistance which have developed in recent years (Harris et al., 1972; Clower et al., 1975) the losses may now have doubled.

Although the principal and preferred food plant of H. zea is corn (Brazzel et al., 1953; Hardwick, 1965; Snow and Brazzel, 1965) extensive damage is also incurred by cotton, tomato, and leguminous plants such as peas. Damage to cotton and tomato crops is, in fact, of such magnitude that on these crops H. zea is referred to as the cotton bollworm and tomato fruitworm, respectively. Although H. virescens has a somewhat

reduced host range [Brazzel et al. (1953) indicated its complete absence from corn over a 2-year study], it is nonetheless of immense importance as a pest of both cotton and tobacco, and is very likely of more importance on cotton than H. zea due to its genetic propensity for the development of insecticide resistance (Harris et al., 1972; Clower et al., 1975). It is probably safe to consider H. zea and H. virescens combined as the most serious pest threat to cotton in the United States today.

Heliothis subflexa has apparently not been recorded as a pest species, and unlike its close relatives has a more confined diet, feeding solely on species of the genus Physalis (Brazzel et al., 1953; Roach, 1975).

Reproductive Biology: Multiple Mating

As among other noctuid species (Byers, 1978) these 3 Heliothis species commonly engage in multiple mating (Callahan, 1958a; Hardwick, 1965; Hendricks et al., 1970), the propensity for which increases with successive generations in laboratory stocks (Proshold and Bartell, 1972; Raulston et al., 1975). Levels of multiple mating in natural populations of H. zea have been found to be 41.7% (Callahan, 1958a), and 33.8% (Hendricks et al., 1970), while a slightly higher value of 45.9% has been reported for H. virescens (Hendricks et al., 1970). Although no data are available regarding natural supernumerary matings in H. subflexa, laboratory tests conducted by Proshold and LaChance (1974) and Pair et al. (1977) indicate that such are common in this species also. Of the several

benefits attributed to multiple mating in Lepidoptera (see Byers, 1978) 2 appear important in these heliothids. First, as females are apparently capable of assessing the genetic adequacy of an initial mating (Proshold and LaChance, 1974; Pair et al., 1977) and as mated females are less receptive than are virgins to additional mating for a period of ca. 3 days after an initial mating (Raulston et al., 1975), multiple mating ensures that a high percentage of eggs are fertilized, thereby limiting the waste of gametes. Second, multiple mating increases the genetic diversity of the offspring of a single female thereby increasing the phenotypic variation and potentially increasing the progeny's fitness.

Interspecific Hybridization

Although broadly sympatric with both H. virescens and H. subflexa, H. zea is effectively isolated from both by structural differences in the genitalia (see Callahan 1958a; Callahan and Chapin, 1960; Hardwick, 1965, 1970). It should not, however, be interpreted that attempted mating between these species does not occur, because Callahan (unpublished but cited by Hardwick, 1965) has found one pair of H. zea and H. virescens locked in copula in the field, and Shorey et al. (1965) reported interspecific copulations with similar results in laboratory cross-mating studies under no-choice conditions. Further, unlike H. subflexa and H. virescens (see below) H. zea is mechanically isolated from its much closer relatives, Helicoverpa (Heliothis) armigera (Hbn) and Helicoverpa (Heliothis) punctigera (Wllgn) (Hardwick, 1965). In fact, of the 115 no-choice hybridization tests carried out between H.

zea and the other 2 helicoverpids, only a single pair (H. armigera females X H. zea males) produced fertile eggs and viable offspring and of these the great majority were infertile (Hardwick, 1965).

Unlike H. zea the genitalia of H. virescens and H. subflexa are sufficiently similar to pose no mechanical barrier to interspecific hybridization. Although early attempts to hybridize these species failed (Brazzel et al., 1953), Laster (1972) was able to obtain offspring from H. subflexa female X H. virescens male crosses. Subsequently Proshold and LaChance (1974) succeeded in obtaining progeny from both H. subflexa female X H. virescens male and H. virescens female X H. subflexa male crosses. Hybrid features of the former cross include: a slight sexual dimorphism in larval development times (Laster, 1972), the entry of ca. 40% of the female pupae into an extended diapause (Proshold and LaChance, 1974), reduced mating, production of fewer eggs, and of major importance, the production of essentially but not fully sterile males (Laster, 1972; Proshold and LaChance, 1974). Interestingly, although male sterility is maintained in successive backcross generations with H. virescens males (Laster et al., 1976), backcrossing with H. subflexa males restores fertility within 2 generations (Karpenko and Proshold, 1977). Distinct features of the reciprocal cross (H. virescens female X H. subflexa male) are much less obvious as none enter diapause and mating occurred as often as among parental stocks in studies carried out by Proshold and LaChance (1974). Further, although infertility occurred in both sexes it was not complete and fertility could be restored with successive backcrossing to either parent (Proshold and

LaChance, 1974; Karpenko and Proshold, 1977). In both hybrid stocks the number of chromosomes is reduced from the 31 pairs found in the parental species to 20-28 bivalents (Proshold and LaChance, 1974).

Proshold and LaChance (1974) contended that the prominent male sterility within both hybrid groups was the result of an inability to transfer eupyrene sperm, perhaps due to chromosome desynapsis. Subsequently, it was found that the number of eupyrene sperm bundles transferred was similar to those transferred in conspecific matings by the parent species, and that the sterility resulted from the inability of the sperm bundles to break up in the spermatophore and from abnormalities in the axial filaments of the sperm (Proshold et al., 1975; Richard et al., 1975). Further, as males from backcross generation 35 do not show chromosome desynapsis, it is unlikely that chromosome deviations are the cause of sterility (Karpenko and Proshold, 1977).

Interspecific Variations

Although Sluss et al. (1978) found low interpopulation variability in allozyme allele frequencies of H. virescens, there is probably considerable interspecific variation, particularly in behavioral characters, due to the broad range and high adaptability of this species. The best evidence for this comes from differences observed between laboratory stocks (Laster et al., 1977), laboratory and wild strains (Raulston et al., 1975; Raulston et al., 1979), and hybrid laboratory X wild stocks (Young et al., 1975). Major characters involved include: mating propensity, oviposition periods and reproductive periods. In fact the 2 h difference

in mating periods between Texas laboratory-reared and Virgin Island wild stocks was of sufficient magnitude to effectively isolate these 2 groups.

Evidence concerning populational differences in H. zea is more concrete. Hardwick (1965) has noted definite morphological differences between the Hawaiian H. zea and continental populations and there is evidence of genetic variation among natural populations (Sell et al., 1975) and laboratory and wild populations (Sluss et al., 1978). Although Sluss et al. (1978) speculate that differences between laboratory and natural populations result from genetic drift within a small isolated laboratory colony, the observations of Raulston (1975) indicate that such differences can occur within 2 generations and are more probably due to environmental parameters.

## Mating Behaviors and Reproductive Periods

As among other noctuid species (Shorey et al., 1968; Teal et al., 1978), H. zea and H. virescens do not become sexually mature immediately after emergence. Although no data are available for H. subflexa, the same condition probably exists. In a behavioral study Callahan (1958b) found that female H. zea began sexual activity on the 2nd night after emergence, while Shorey et al. (1968) found that a substantial percentage mated on the 1st night. Both studies indicated that males were capable of copulation on the 1st night postemergence. Data concerning H. virescens is much more obscure. Although Shorey et al. (1968) reported females mating on the 1st night after emergence, Hendricks

and Tumlinson (1974) found females actively calling on the 2nd night, and Gaston and Shorey (1964) reported maximum sexual attraction occurred on the 4th night, while Gentry et al. (1964) found females unattractive until 5 days old. Although pheromone release is certainly not an absolute indication of reproductive maturity and ability to mate, pheromone release does not generally reach its maximum until gametes are mature (Shorey, 1974). The differences reported by the above workers may be the result of intraspecific differences resulting from laboratory colonization. Male H. virescens reach reproductive maturity on the 1st or 2nd night after emergence (Gentry et al., 1964; Shorey et al., 1968).

The behaviors and postures assumed during pheromone release by H. zea and H. virescens have been described by Callahan (1958b), Agee (1969), Hendricks and Tumlinson (1974), and Mitchell et al. (1974). Agee (1969) also indicated that females wiped their ovipositors on the substrate, an act which has also been reported in the pink bollworm (Leppla, 1972) whereby females deposit pheromone on the supporting surface (Colwell et al., 1978a).

Male precopulatory behaviors have apparently been studied sequentially only in H. zea, and even then only in small cage studies (Callahan, 1958b; Agee, 1969), although the criteria used in bioassay experiments by Shorey and Gaston (1965), McDonough et al. (1970), and Hendricks and Tumlinson (1974) are obviously involved in the ethology of mating. In H. zea the sequence involves: antennal movement and wing fluttering, circular ambulation, flight, extension of the claspers, landing and an

ambulatory approach close to the side of the female, continued wing vibration, and a grab for the female ovipositor which terminates in copulation (see Callahan, 1958b; Agee, 1969).

The diel mating periodicity of the 3 species has been studied both in the laboratory (Callahan, 1958b; Gentry et al., 1964; Shorey and Gaston, 1965; Hendricks and Tumlinson, 1974), and field (Goodenough and Snow, 1974; Hendricks and Tumlinson, 1974; Mitchell et al., 1974; Raulston et al., 1975, 1979; Tingle et al., 1978). These studies indicate the diel periodicities as follows: H. zea 1-10 h in the scotophase (peak at 4 h), H. virescens 3-9 h (peak at 6 h), H. subflexa 1-8 h (peak at 4 h). In addition, studies by Raulston et al. (1975) indicate that mated females containing sperm have a peak period of mating that occurs 1 h later than that of virgin females. A further study (Raulston et al., 1976) has also shown a difference of 2 h between a wild strain from the Virgin Islands and a laboratory strain from Texas.

Sex Pheromone Glands and Production

The sex pheromone gland of both H. zea and H. virescens has been described as a complete ring of collumnar and cuboidal class I epidermal gland cells (see Noirot and Quennedey, 1974) situated in the intersegmental membrane between abdominal segments 8 and 9 (Jefferson et al., 1968). Unlike H. zea, which shows a rapid production of pheromone prior to emergence, H. virescens emerges before any appreciable amount of pheromone is produced (Shorey et al., 1968). The rise in the pheromone concentration is, however, of such magnitude that by the 1st night after

emergence both H. zea and H. virescens produce about the same amounts (Shorey et al., 1968).

The first evidence of the production of a volatile sex pheromone by female H. virescens was reported by Gentry et al. (1964) using extracts of 5-day-old females and abdomen tips. Similarly, crude abdomen tip extracts of both H. virescens and H. zea elicited male ambulatory and flight behaviors in laboratory bioassays. Berger et al. (1965), failed to obtain male responses from either crude extracts or airborne collections, and suggested that, as male responses were obtained from gas chromatographic (GC) fractions, crude extracts were masked by the presence of inactive contaminants within the gland. This masking was subsequently negated by Shorey and Gaston (1967) in tests using both crude extracts and GC fractions. Interestingly, the extracts used in Shorey and Gaston's study failed to elicit close-range copulatory behaviors, while excised ovipositors did, indicating that perhaps the complete pheromone blend was not extracted.

## Sex Pheromone Chemistry

McDonough et al. (1970) were first to report the isolation and identification of behavior-influencing chemicals from H. zea. Utilizing the same bioassay techniques used in Shorey's studies these workers concluded that (E)-7-tetradecen-1-ol acetate and (E)-7-tetradecen-1-ol were active components and indicated that several 14-carbon alcohols and acetates could also be involved. As discussed by Mayer and McLaughlin (1975), however, evidence for their actual role as sex pheromones is

tenuous. (Z)-9-Tetradecen-1-ol formate, a compound subsequently found to be an effective mimic of one of the actual components of the pheromones of both species [(Z)-11-hexadecenal] (Mitchell et al., 1975, 1978), was postulated as a pheromone of H. zea and, in combination with (E)-9-tetradecen-1-ol, the pheromone of H. virescens (Jacobson et al., 1972). Subsequent field testing (Hendricks, unpublished but cited by Tumlinson et al., 1975; Mitchell et al., 1978), however, indicated that neither component was as effective as crude abdominal washes. Subsequently, Roelofs et al. (1974) isolated and identified (Z)-11-hexadecenal and (Z)-9-tetradecenal as pheromone components of H. virescens and (Z)-11-hexadecenal from H. zea. Tumlinson et al. (1975) confirmed the presence of (Z)-11-hexadecenal and (Z)-9-tetradecenal in H. virescens, finding a ratio of 16:1 within the insects. However, because synthesized mixtures of the H. virescens pheromone were not as effective as crude abdominal extracts in attracting males in field cage tests these workers speculated that other chemicals were also produced.

The presence of other chemicals in the gland of both H. zea and H. virescens has indeed been reported recently (Klun et al., 1980a,b). According to Klun et al., in addition to (Z)-11-hexadecenal traces of (Z)-9-hexadecenal, (Z)-7-hexadecenal, and hexadecanal are present in the pheromone of H. zea, while in addition to these chemicals the pheromone of H. virescens contains minute amounts of tetradecanal, (Z)-9-tetradecenal, and (Z)-11-hexadecen-1-ol. As trap catch is significantly increased by the presence of these compounds in baits (Klun et al., 1980a,b), certain of these compounds are undoubtedly principal components of the pheromones of these species. Bioassays used by both

Roelofs et al. (1974) and Tumlinson et al. (1975) relied heavily upon sustained flight and other cues attributed to primary pheromone components (see Roelofs and Carde, 1977); thus, it seems probable that the newly identified compounds are secondary, or close-range components. Unfortunately, it is unknown if all or just certain of these chemicals are necessary for maximum attraction and reproductive isolation between these 2 species. To date none of the pheromone components of H. subflexa have been reported.

Interspecific Attraction and Stimulation

Early evidence based on behavioral studies indicated that considerable cross-attraction occurred between H. virescens and H. zea (Shorey et al., 1965). Cross-attraction was so prominent in the above study that the authors considered mechanisms other than pheromone to be involved in reproductive isolation. All studies on the pheromone chemistry of these species have shown the presence of at least one common component. Interestingly, studies on the oldworld bollworm, H. armigera, also indicate the presence of (Z)-11-hexadecenal and (Z)-9-hexadecenal (Nesbitt et al., 1979, 1980) common to the pheromones of both H. zea and H. virescens and perhaps (Z)-9-tetradecenal in common with H. virescens (Gothilf et al., 1978). The use of common chemical components by closely related species is well known (Roelofs and Carde, 1974; Carde et al., 1977), and appears to be of considerable value in phylogenetic studies (Roelofs and Comeau, 1969). Among tortricid moths, for which considerable amounts of data on the component makeup of many

species are available, many species use the same chemical compounds but rely on component ratios as a mechanism of isolation (Carde et al., 1977). As indicated by the work of Klun et al. (1979), specificity within these heliothids does not rely solely upon component ratios, but also on the sequential addition of minor components to the pheromone blend. Because males invest considerable amounts of energy during upwind orientation, one draws the conclusion that certain of the trace chemicals in the H. virescens pheromone might inhibit upwind flight by male H. zea. Further, as the presence of virgin female H. zea and H. virescens in the same electric grid trap significantly reduces male attraction in both species (Haile et al., 1973), it is apparent that, when in close association, the pheromones of both species or appropriately combined ratios thereof are inhibitory to males. Similar cases of interspecific inhibition among other noctuids have been described by Steck et al. (1977).

Semiochemicals for the Control of H. zea and H. virescens

Surprisingly, in view of our limited knowledge of the mechanics of pheromone communication in these species, semiochemicals play major roles, both directly and indirectly, in many control programs.

The use of both virgin females and synthetic attractants in population monitoring is common (see Goodenough and Snow, 1973; Roach, 1975). Although there is no literature available on extensive mass-trapping using the pheromones of H. zea and H. virescens it has been alluded to (Mitchell et al., 1974). Currently, the most promising use

of semiochemicals in the direct control of these species is in mating disruption. In studies on multi-species disruption using (Z)-7-dodecen-1-ol acetate (pheromone of Trichoplusia ni (Hübner)) and (Z)-7-hexadecen-1-ol acetate (an attractant of the pink bollworm), Kaae et al. (1972) achieved considerable success in the disruption of both H. zea and H. virescens, particularly with looplure. Also, recent studies based on electroantennogram data (Priesner et al., 1975; Mitchell et al., 1975, 1976, 1978) have shown that atmospheric permeation using (Z)-9-tetradecen-1-ol formate is a highly effective disruptant for both species. Further, Mitchell et al. (1978) have shown that (Z)-11-hexadecenal is equally effective in disruption and conclude that the formate probably substitutes for the aldehyde at the level of the antennal receptor. Interestingly, (Z)-7-dodecen-1-ol formate, a very similar chemical to (Z)-9-tetradecenal, is not effective in disruption, and when H. virescens females are used as bait in an area permeated with this formate considerable numbers of H. zea males are trapped (Mitchell et al., 1978). Because (Z)-9-tetradecen-1-ol formate has been used effectively with mating disruptants of other species it promises to be of considerable value in the future (Mitchell et al., 1976).

In addition to the direct use of semiochemicals in the control of both H. zea and H. virescens, programs using the sterile-release technique (see Knipling, 1970) rely heavily upon the attraction and subsequent mating of native and released insects. Recently, based on the inherited sterility of backcross progeny resulting from H. subflexa female X H. virescens male hybrids, Laster et al. (1976) and Parvin et

al. (1976) have proposed such a program for the control of H. virescens. Although an impressive and potentially very effective control proposal, the original Laster-Parvin model contained several inconsistencies that have been discussed and corrected by Makela and Huettel (1979). From a chemical communication standpoint several factors appear unresolved in the Laster-Parvin model. Although an investigation by Laster et al. (1978) indicated that backcross (BC) and H. virescens females were equally effective in the long-distance attraction of H. virescens males, no comparison was made with virgin wild females, nor were close-range copulatory behaviors critical to mating studied (see Roelofs and Carde, 1977). Under natural unrestricted conditions wild males may exhibit considerable choice favoring wild virgin females due to: 1) differences in the pheromonal bouquet of laboratory-reared stocks due to dietary differences (see Hendricks et al., 1977), differences in the place of origin of the laboratory stocks (see Roelofs and Carde, 1974; Laster et al., 1977), or differences resulting from hybridization which effect close-range copulatory behaviors; 2) the evolution of close-range courtship behaviors in laboratory stocks that differ significantly from those of wild stocks due to high levels of selection resulting from laboratory colonization (see Raulston, 1975; Chambers, 1977; Sluss et al., 1978). Although many of these factors appear to have been tested by Raulston et al. (1979), these authors indicate that their tests were not carried out in the peak period of virgin mating and, as mated females are less active sexually for some time after mating, the continuously active males may have settled for the next best thing, the BC

females (a similar theory was proposed for the attraction of males to pheromone traps by these authors). Raulston et al. (1979) also indicated that BC males were not competitive with H. virescens males in mating with wild females, a point which supports the above and indicates that further research into pheromonal communication between these insects is warranted.

## Research Aims

Obviously there are inconsistencies and gaps in our knowledge of the biology of H. virescens, H. subflexa, and H. zea. Authors, even within the same research group, report conflicting results, but nevertheless studies appear to take for granted basic facts that have not been proven conclusively. This is all too evident in studies on pheromone communication among these species. For example, a total of 7 compounds have been identified as behavioral-modifying chemicals for H. zea by 4 groups of researchers, 9 compounds for H. virescens, and none for H. subflexa. Additionally, for those which have been identified from the pheromone glands we have no idea which are involved in upwind amenotaxis and which are important in close-range precopulatory behaviors, or in fact which are actually released as volatiles from the gland surface. Further, we have no conception as to the effect of hybridization on pheromone-induced behavior within the group, and, as control programs are fast moving toward the use of sterile hybrids in control, such knowledge is a necessity.

The present study was undertaken to evaluate several aspects of the sex pheromone mediated biology and chemistry of H. virescens and H.

subflexa in an attempt to provide the basics necessary for the development of effective semiochemical control programs and to assess the effects of sex pheromones on the reproductive isolation of these species.

In order to accomplish these goals the following studies were undertaken: 1) an analysis of the reproductive behavior of H. virescens under laboratory conditions; 2) an investigation of the sites of sex pheromone production in the ovipositor of H. virescens; 3) a behavioral and electrophysiological analysis of extracts obtained from these sites; 4) a behavioral analysis of the semiochemically induced interactions occurring between H. virescens and H. subflexa; and 5) the identification of a sex pheromone of H. subflexa and assessment of different blends of its components in field trapping studies.

# CHAPTER II
# ANALYSIS OF THE REPRODUCTIVE BEHAVIOR OF *HELIOTHIS VIRESCENS* (F.) UNDER LABORATORY CONDITIONS

## Introduction

Due to its genetic propensity for developing resistance to insecticides (Harris et al., 1972; Clower et al., 1976), *H. virescens* has become the subject of numerous studies using potential alternative methods of pest suppression. One such method, population reduction by infusion of hybrid sterility into natural popualtions, shows considerable promise, being both environmentally safe and effective over extended periods of time (Laster et al., 1976). The success of this approach depends upon random mating between native populations of *H. virescens* and introudced backcross individuals obtained through laboratory hybridization of *H. virescens* and its sibling species, *H. subflexa*.

Among sympatric, closely related species of Lepidoptera, reproductive isolation occurs prior to gamete investment, being controlled to a great extent by sex pheromones and related behaviors (Roelofs and Carde, 1974). Although early studies suggested the use of a single chemical component pheromone (Butenandt et al., 1959; Roelofs and Arn, 1968), recent work has indicated that few, if any, Lepidoptera rely upon such simple systems. In addition to the species specificity imparted by multicomponent pheromone blends and ratios acting at a distance (Carde et al., 1977), it appears that certain minor components acting either alone or in concert with other components are responsible for maximizing the behavioral events comprising courtship (Roelofs and Carde, 1977).

Hence it is of utmost importance that the sequence of behavioral events required for successful intraspecific mating be documented prior to the ascribing of roles to the pheromone components or attempting to enhance interspecific mating success via pheromonal means.

Although courtship studies have been made on H. zea (Callahan, 1958a,b; Agee, 1969), these studies were not primarily concerned with the role of sex pheromones, and as shown elsewhere (Grant and Brady, 1975; Grant et al., 1975), behaviors required for successful mating by one species cannot be extended to other closely related species. This study reports the results of laboratory studies on the precopulatory behaviors of H. virescens as a prelude to work on the reproductive interactions between H. virescens and H. subflexa.

## Methods and Materials

### Rearing and Adult Holding

Heliothis virescens used for all studies were obtained as pupae from laboratory stocks maintained at USDA facilities in both Oxford, NC, and Stoneville, MS. All insects were allowed to emerge under test conditions and in isolation from members of the opposite sex. Newly emerged adults, collected daily, were housed in 30 X 30 X 30 cm plexiglass cages for at least 2 days prior to testing to ensure reproductive maturity. All cages were provided with a 10% sucrose solution for nutrient. The upper limit for testing was set at 8 days postemergence. Tests were conducted during the dark phase of a reverse 16:8 light:dark cycle at a temperature of 20(±1)°C, and relative humidities of 54(±2)% during the day and 60(±2)% during the night. Test individuals of both

sexes were selected on the basis of good appearance and physiological vigor, as indicated by sustained flight after being startled during the photophase preceding each test.

Bioassays

Two assay systems were employed to provide data on both precourtship and close-range courtship behaviors. The 1st, used in the analysis of male activation, orientation, and initial analysis of courtship interactions, consisted of a 1.5 X 0.5 X 0.5-m plexiglass wind tunnel through which air was pulled at a constant rate. Groups of 5 females were placed on a tobacco plant in the upwind end of the tunnel during the premating period of the scotophase (Tingle et al., 1978). Female behavior was monitored throughout the mating period. Individual males were placed in release cages positioned above the body of the tunnel and 10 cm from the downwind end at least 15 min prior to being lowered into the tunnel. The largest possible pheromone plumal area was estimated with titanium tetrachloride fumes emitted from cotton wicks positioned to approximate the outermost edges of the tobacco plant. A light level of 2 lux, to which the insects had been entrained during holding, was found to be sufficient for observations when augmented with a diffuse red-filtered flashlight during close-range studies. The comments of an observer were recorded on audio-cassette tapes and later transcribed.

The 2nd assay system was used to observe courtship interactions and consisted of a 20 X 10 X 10-cm plexiglass cage having a 4-$cm^2$ door at one end to admit insects. Groups of 3-4 females were placed in the

chamber during the photophase preceding each test, and males were released individually when females had been observed calling for a 5-min period. Behaviors were recorded on video-tape using a JVC© cassette recorder and a Panasonic WV-1050® camera with spectral sensitivity between 400-800 nm (minimum light intensity for camera sensitivity = 0.5 lux), a neutral density remote-control zoom lens and a remote-control pan-tilt device. Tapes were transcribed from a television monitor using both stop action and standard speed modes.

Statistical Anlysis

Frequencies of the observed behaviors were tabulated in 1st order, preceding-following, behavior transition matrices, and comprehensive ethograms were devised. Chi-square values of all cells with probabilities >0 were calculated according to common techniques (Stevenson and Poole, 1976). Since a successful mating included only a single approach and clasp, and since self-perpetuated acts were considered single events, $\chi^2$ comparisons were based on 56 degrees of freedom. Individual transitions with observed frequencies greater than the expected value were considered significantly greater than chance if (observed - expected) ÷ (expected)$^{.5}$ values were greater than $(\chi^2_{0.05,\ 56df})^{.5} \div 56^2$ (Bishop et al., 1975; Fagen and Young, 1978). Standard normal deviates were calculated for successful courtship sequences and applied to a binomial test for individual transitions (Siegel, 1956). Transitions with deviates yielding probabilities of $p < 0.05$ were used in construction of an ethogram to indicate the most probable courtship interaction sequences resulting in successful mating.

## Observations and Discussion

### Female Precourtship Behavior (N = 75)

In common with other species of Lepidoptera (Agee, 1969; Fatzinger and Asher, 1971; Barrer and Hill, 1977; Teal and Byers, 1980), distinct phases of calling (sex pheromone release) were obvious in our study. One phase, indicating the initiation of female sexual activity, was marked by short bouts of calling separated by periods of ambulation, wing fanning, and flight. Although the glandular surface was normally retracted during interruptions, some females were seen with their ovipositors extended during periods of flight and ambulation. The 2nd phase of calling was preceded by a period of scent marking ($p = .73$) during which time females dragged the ventral portion of the ovipositor on the supporting substrate. As males will land and search a scent-marked area, often exposing their hairpencils, after removal of the female, such pheromone deposits appear to aid in the chemical elicitation of male reproductive behaviors. Following this, females entered a period of quiescent calling during which they remained relatively stationary and held their wings in a "V" at ca. 45° to the body. Unlike female H. zea (Agee, 1969), wing vibration by female H. virescens during this time was sporadic and of short duration ($p = .08$) in the small populations tested.

Interestingly, these 2 phases of calling are fairly well correlated with sexual receptivity. Females calling for short sporadic bouts actively reject males that are committing appropriate behaviors early in the courtship sequence. However, females calling for prolonged bouts

tend to reject males because of incorrectly committed male behaviors in the latter part of the sequence (Table 1). Because a male bias in the operational sex ratio probably exists under natural conditions (Teal et al., 1978), rejections during the initial phase of calling would probably not affect an individual female's chances for successful mating on a given night. Further, as the mating periods of H. zea and H. subflexa [both known to enter into interspecific copulation with H. virescens in the laboratory (Shorey et al., 1965; Laster, 1972; and see Hardwick, 1965 for a naturally occurring case)], do marginally intersect the early part of the H. virescens calling period (Tingle et al., 1978), the reduced sexual receptivity during early discontinuous calling bouts may serve to isolate female H. virescens behaviorally during chance encounters with related species.

As a result of sporadic calling during the early bouts, effective chemical signaling occurs primarily during the prolonged quiescent calling period. This is quite different from that of many other noctuid species, which exhibit discontinuous patterns of calling throughout the mating period (Sower et al., 1971; Marks, 1976; Swier et al., 1977). Since constant shifting by calling females coupled with stationary scent marks would tend to confuse orienting males, it would seem advantageous for females to remain within a restricted area during calling and enhance the orientation signal by both calling and depositing a relatively concentrated scent mark. Support for this hypothesis comes from the calling behaviors of 2 other species commonly occurring at high population densities, H. zea and Pectinophora gossypiella (Saunders),

Table 1.--Courtship rejections of H. virescens males as indicated in small cage laboratory videotape studies.

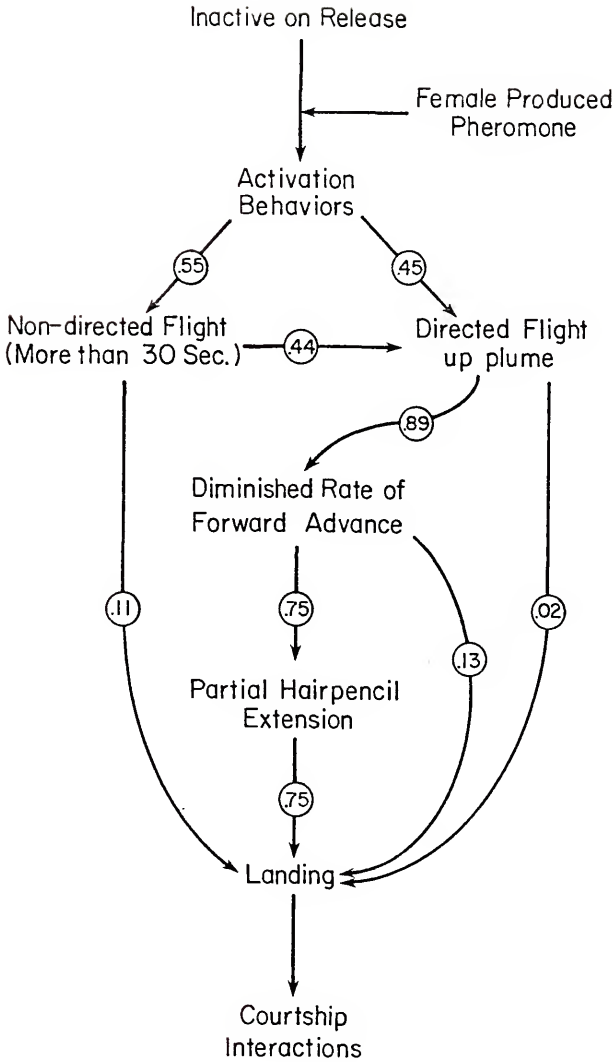
| Courtship breakdown behavior | | No. of rejections | Probability | Sex rejecting | Reasons for rejection |
|---|---|---|---|---|---|
| Approach | 1 | 2 | 0.08 | Female | Female not receptive (short bouts) |
| | 2 | 1 | 0.04 | - | Male fails to continue |
| Movement under wing | 1 | 2 | 0.09 | Female | Female not receptive (short bouts) |
| | 2 | 1 | 0.04 | Male | Improper female cue? male reorients |
| Hairpencil | | 2 | 0.09 | Female | Male too far away from female |
| Movement parallel | 1 | 7 | 0.3 | Female | Male fails to get parallel, clasp missed. |
| | 2 | 3 | 0.13 | Female | Female not in quiescent calling bout |
| | 3 | 1 | 0.04 | Male | Male moves away, no clasp. |

which call for extended periods near scent marks (Agee, 1969; Colwell et al., 1978a,b).

Male precourtship behaviors (N = 76)

Of 76 males exhibiting reproductive behaviors in the 1.5-m flight tunnel, 44 began from rest and committed common pheromonally mediated activation behaviors (Fig. 1) (see Bartell, 1977). The remainder were active prior to or become active while being lowered into the plume. A possible reason for immediate activity in our tests was movement during and prior to release. However, a constant basal activity level of ca. 20% has been noted in bioassay studies with synthetic and glandular odorants (Teal et al., unpublished data). The existence of a naturally occurring, non-mating activity period is common among many moth species (Carde et al., 1975; Baker et al., 1976; Marks, 1976), and judging from disparities between time intervals for significant trap captures using blacklight and female-baited traps (Graham et al., 1964; Goodenough and Snow, 1973), such a premating activity period is indicated for H. virescens. Hence, it is probable that a major portion of the natural male population encounters pheromone stimuli while in flight (Sparks et al., 1979), and the significance of the various activation behaviors listed below are therefore indeterminant. Although wing elevation, vibration, and antennal movement were similar to those described for H. zea (Agee, 1969), no H. virescens males were seen extending and wiping their claspers on the substrate prior to flight. Antennal cleaning with the epiphysis of the foreleg (Callahan and Carlysle, 1971) and extension

Figure 1. Precourtship reproductive behaviors of initially inactive male H. virescens (n = 44). Males are not necessarily successful during courtship. Values indicate probabilities.


Inactive on Release
Female Produced Pheromone
Activation Behaviors
.55
.45
Non-directed Flight (More than 30 Sec.)
.44
Directed Flight up plume
.89
Diminished Rate of Forward Advance
.11
.75
.13
.02
Partial Hairpencil Extension
.75
Landing
Courtship Interactions

of the proboscis were also common (p = 0.5), as was a period of random ambulation during preflight wing fanning (p = 0.64). Although these activation behaviors are of benefit in analysis of interspecific pheromone communication, I have, for the reason above, not elaborated them for analysis (Fig. 1).

Initially active moths underwent a period of erratic flight, bumping into the walls, moving past calling females (sometimes within 4-5 cm), and stopping frequently. Preceding upwind taxis, these moths generally performed a series of horizontal and vertical sweeps through the downwind 1/2 of the plumal area. A similar period of aerial searching was also observed during 55% of the tests involving initially inactive males. In common with other species (Kennedy and Marsh, 1974; Farkas and Shorey, 1974), flight upwind toward calling females was marked by a series of tightening zigzags approximating plume width. At a distance of 5-15 cm from the calling female, the rate of forward advance was distinctly slowed (p = 0.89), and in some instances halted momentarily as males entered a "choice" situation. At this point males made the "decision" to continue or to reorient from downwind. Continuance in all cases was marked by a momentary hairpencil exposure in which the hairpencils were extended to ca. 1/2-3/4 of their length and landing from 2-4 cm to the lower left (p = 0.46) or right (p = 0.54) of the female. As males undergoing random flight or aerial searching rarely entered into courtship during encounters with receptive females (p = .03), a period of close-range tactic behavior, during which males perceive sufficient stimuli to release continued reproductive behaviors,

appears to be necessary. This close-range male orientation and discrimination phase is apparently necessary for reproductive isolation because, although male H. virescens will move toward calling H. subflexa females from a distance, the majority are incapable of either finding females when at close range or completing courtship.

Courtship Interactions

Although flight tunnel assays were useful in outlining the more obvious steps in male courtship, a considerable number of male-female interactions went unobserved, necessitating the use of video-tape studies. Video analysis of 30 successful matings revealed a highly variable series of interactions (Fig. 2, Table 2), in which only one transition with an observed frequency greater than the expected result (female wing fanning ⇒ partial hairpenciling) was not significantly greater than that expected by chance. However, results of a binomial test of individual transitions indicated that a common trend was present (Fig. 3). A male commonly began the sequence by approaching and moving under a female's wing, but unlike H. zea (Agee, 1969) did not antennate the ovipositor. At this point, the female would normally fan her wings. The male continued his forward advance, partially exposing his hairpencils in the process, until his head was approximately parallel with the female's. Having achieved this position, the male commonly turned to face the female and, in 45% of the matings, the female also turned toward the male. At this point, the male hairpenciled and clasped the female's genitalia. Interestingly, male wing fanning, a mechanism used

Figure 2. Comprehensive ethogram of 30 successful matings. Values indicate probabilities of performance of a pair of behaviors sequentially.

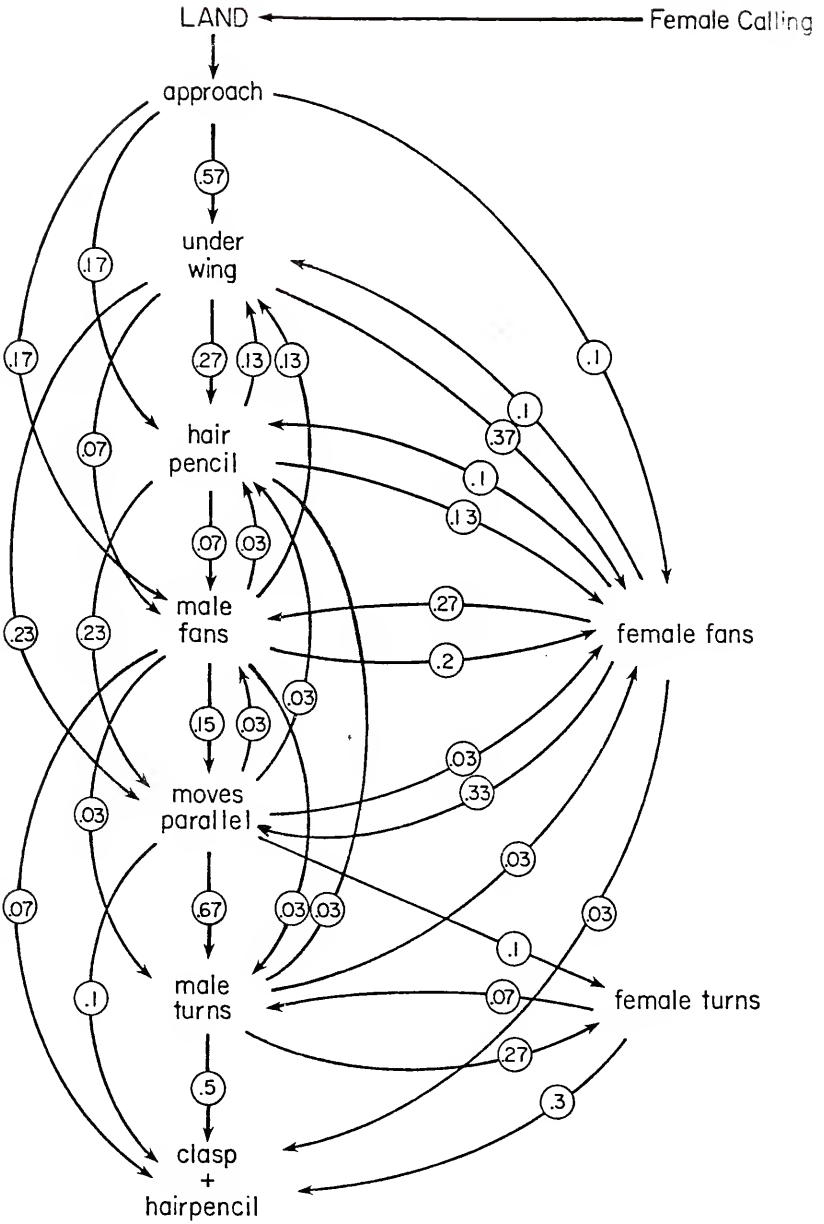

LAND
Female Calling
approach
.57
under wing
.17
.17
.27
.13
.13
.1
.1
.37
.07
hair pencil
.1
.13
.07
.03
.27
male fans
female fans
.23
.23
.2
.03
.15
.03
.03
.33
moves parallel
.03
.03
.07
.67
.03
.03
.03
.1
.1
male turns
.07
female turns
.27
.5
.3
clasp + hairpencil

Table 2.--First-order behavioral transitions in successful matings of H. virescens observed in laboratory wind tunnel studies (N = 33).

| Preceding Behavior | Following Behavior: Approach | Under ♀ wing | ♀ Fans wings | ♂ Fans wings | Variable hairpencil | ♂ Moves parallel | ♂ Turns to ♀ | ♀ Turns to ♂ | Hairpencil and clasp | Σ |
|---|---|---|---|---|---|---|---|---|---|---|
| Approach | (obs)[a] | 17 | 3 | 5 | 5 | 0 | 0 | 0 | 0 | |
| | (exp) ___ | 5.45 | 4.87 | 3.70 | 3.51 | - | - | - | - | 30 |
| | ($\chi^2$) ___ | 24.48 | ___ | 0.46 | 0.63 | - | - | - | - | |
| | (Z)[b] | 4.95 | ___ | 0.67 | 1.04 | - | - | - | - | |
| Under | (obs) | ___ | 11 | 2 | 8 | 7 | 0 | 0 | 0 | |
| ♀ | (exp) ___ | ___ | 4.49 | 3.41 | 3.20 | 5.21 | - | - | - | |
| wing | ($\chi^2$) ___ | ___ | 9.49 | ___ | 7.20 | 0.61 | - | - | - | 28 |
| | (Z) | ___ | 3.07 | ___ | 2.68 | 0.78 | - | - | - | |
| ♀ | (obs) | 3 | | 8 | 3 | 10 | 0 | 0 | 1 | 25 |
| Fans | (exp) ___ | 4.40 | ___ | 2.99 | 2.83 | 4.56 | - | - | 4.72 | |
| wings | ($\chi^2$) ___ | ___ | ___ | 8.39 | 0.01 | 6.49 | - | - | - | |
| | (Z) | ___ | ___ | 2.90 | 0.10 | 2.55 | - | - | - | |
| ♂ | (obs) | 4 | 6 | | 1 | 5 | 1 | 0 | 2 | 19 |
| Fans | (exp) ___ | 2.19 | 2.88 | ___ | 2.07 | 3.34 | 2.76 | - | 3.45 | |
| wings | ($\chi^2$) ___ | 1.50 | 3.88 | ___ | ___ | 0.83 | - | - | - | |
| | (Z) | 1.22 | 1.84 | ___ | ___ | 0.81 | - | - | - | |
| Variable | (obs) | 4 | 4 | 2 | | 7 | 1 | 0 | 0 | 18 |
| hairpencil | (exp) ___ | 3.04 | 2.71 | 2.06 | ___ | 3.14 | 2.60 | - | - | |

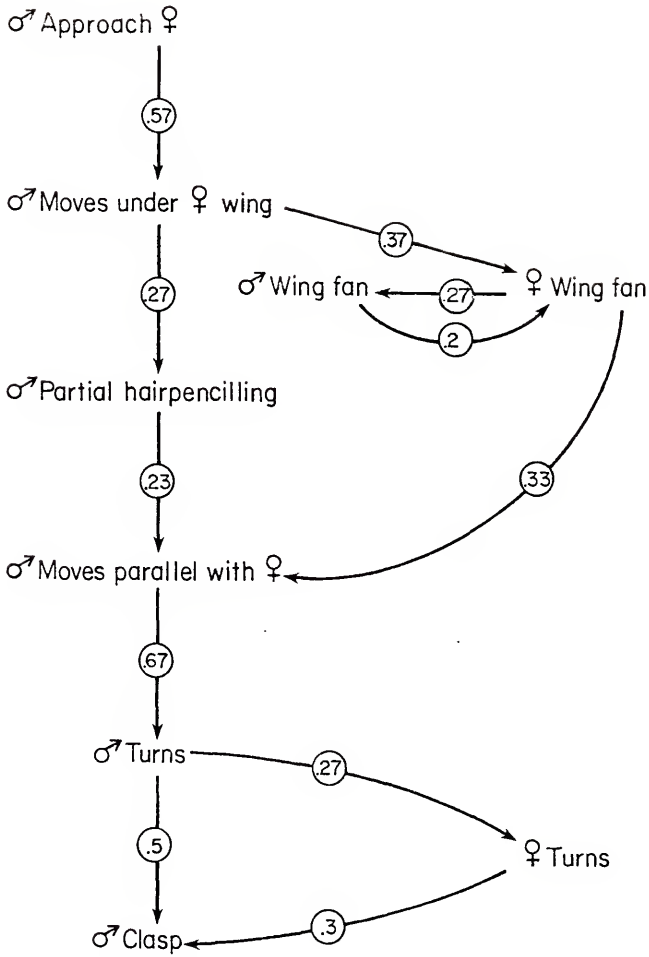
Table 2.--continued.

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | ($\chi^2$) | | 0.30 | 0.61 | ___ | ___ | 4.75 | - | - | - | |
| | (Z) | | 0.55 | 0.78 | ___ | ___ | 2.18 | - | - | - | |
| ♀ | (obs) | | 0 | 1 | 1 | 1 | | 20 | 3 | 3 | 29 |
| Moves | (exp) | | - | 4.68 | 3.55 | 3.37 | | 4.49 | 2.06 | 5.61 | |
| parallel | ($\chi^2$) | ___ | - | - | - | - | ___ | 53.57 | 0.43 | - | |
| | (Z) | | - | - | - | - | ___ | 7.32 | 0.66 | - | |
| ♂ | (obs) | | 0 | 0 | 1 | 0 | - | | 8 | 15 | 24 |
| Turns | (exp) | ___ | - | - | 2.85 | - | - | ___ | 1.65 | 4.50 | |
| to ♀ | ($\chi^2$) | ___ | - | - | - | - | - | ___ | 24.44 | 24.50 | |
| | (Z) | | - | - | - | - | - | - | 4.943 | 4.95 | |
| ♀ | (obs) | ___ | 0 | 0 | 0 | 0 | 0 | 2 | | 9 | 11 |
| Turns | (exp) | | - | - | - | - | - | 1.52 | ___ | 1.91 | |
| to ♂ | ($\chi^2$) | ___ | - | - | - | - | - | 0.14 | ___ | 26.32 | |
| | (Z) | | - | - | - | - | - | 0.38 | | 5.13 | |
| Hairpencil | | | | | | | | | | | |
| and clasp | | | - | - | - | - | - | - | - | - | |
| Σ | | 0 | 28 | 25 | 19 | 18 | 29 | 24 | 11 | 30 | 184 |

[a]Obs = observed value; exp = expected value.

[b]Z = standard normal deviate.

Figure 3. Most frequent behavioral paths in successful copulations based on standard normal deviates having probabilities <0.05 in a binomial test. Male wing fanning is offset as it does not appear to interact directly with other male behaviors. Values are probabilities given in Fig. 2.


♂ Approach ♀
.57
♂ Moves under ♀ wing
.37
♂ Wing fan
.27
♀ Wing fan
.27
.2
♂ Partial hairpencilling
.23
.33
♂ Moves parallel with ♀
.67
♂ Turns
.27
.5
♀ Turns
♂ Clasp
.3

for scent dispersion of male Grapholitha molesta (Busck) (Baker and Carde, 1979) and Plodia interpunctella (Hübner) (Grant and Brady, 1975), was most often the response to female fanning. Since fanning directs air to the posterior of the male (Baker and Carde, 1979) and away from female H. virescens, it does not appear to serve in scent dissemination in this case. More probably, male fanning is merely the result of female contact and the male's excited behavioral state.

Copulatory Behaviors

Among many lepidopteran species, successful genitalic clasping is followed by an immediate shift to a "heads away" position (Agee, 1969; Grant and Brady, 1975; Barrer and Hill, 1977). However, the immediate shift (i.e., <10 sec) among engaged H. virescens pairs occurred in only 45% of the copulations and was obviously a result of attempted escape by the female. Although both sexes appeared to effect the turn, the major benefit is apparently to the male, as it enables him to extend his hairpencils fully. This is the only time when the hairpencils are fully exposed and pulsed in and out. Since this behavior continued until the female acquiesced, I assume that the hairpencils release a secretion which serves an arrestant function. Further, as females commonly resumed calling and attracted males after courtship breakdowns in which males partially or fully exposed their hairpencils ($p = 0.67$ both assays), the effects of this secretion in inducing a cessation in pheromone release (Hendricks and Shaver, 1975) are clearly short lived.

Courtship Breakdown and Reorientation

In both assay systems, a considerable number of the initial reproductive encounters failed to terminate in successful mating (32/76 flight tunnel; 23/53 video studies), and although males in 72% of the failures monitored in the flight tunnel reoriented, either from downwind ($p = 0.43$) or via ambulation ($p = 0.57$), fewer than 20% were successful in copulating on subsequent attempts. Although males may reorient several times, only 54% of those undergoing ambulatory reorientation after the 1st encounter attempted mating with the same female. Of those reorienting from downwind, only 4 were successful in engaging a female and only one succeeded on the next encounter. Video analysis of courtship failures involving 23 pairs of insects revealed that the majority of breakdowns were due to the failure of a male to move parallel with the female prior to attempted clasping and hairpencil exposure (Table 1), a failure which resulted in these behaviors being performed across the female's abdomen. Females commonly responded by withdrawal of the ovipositor, ambulation, and extensive wing fanning. Although failure on the part of the male accounted for most breakdowns (Table 1), female rejection during the approach, movement under the wing and movement parallel phases was also common ($p = 0.31$). These rejections were the result of encounters involving females which called for short, sporadic bouts. From these data I suggest that the absolute prerequisites for successful mating include: 1) female quiescence during male approach, 2) the male's ability to move parallel with the female prior to hairpenciling and attempting to clasp, and 3) relative quiescence on the part of the female during the clasp attempt.

Behavioral Releasers

The induction of particular reproductive behaviors results from various species-specific cues that cause behavioral release (Grant and Brady, 1975; Baker and Carde, 1979). These cues range from self-induction by the preceding behaviors of the same individual to cases in which both inter- and intraindividual cues moderate transitions (Grant and Brady, 1975; Barrer and Hill, 1977; Baker and Carde, 1979; Castrovillo and Carde, 1980). As among other species, the primary releaser of male reproductive behaviors is the female's sex pheromone blend which, at a minimum, induces orientation. The 1st male-produced cue appears to be a prelanding hairpencil display, which may reinforce female quiescence. Male approach is probably induced by both the presence of the specific pheromone bouquet and the female form. However, preliminary results with a model system similar to that of Shorey and Gaston (1970) suggest that the chemical cue strongly outweighs any visual input. Exposure of the male hairpencils during this period presumably reinforces female quiescence. Upon coming parallel and turning toward the female, the male's forelegs come in close approximation with the female's face. This sequence is of interest in view of Callahan's (1969) description of scent-like scales on the male forelegs of H. zea, and although Callahan found foreleg-amputated males capable of mating, a chemical messenger cannot be ruled out. The final hairpencil display, delivered during the clasp, possibly functions in the maintenance of female quiescence, although since a number of females attempt escape, it is of limited effect.

As discussed by Grant et al. (1975) and Grant and Brady (1975), it is not unlikely that closely related sympatric species sharing common periods of reproductive activity can functionally maintain species isolation solely on the basis of long-distance pheromones. This is particularly true among such sibling species as H. virescens and H. subflexa, which are capable of producing viable, semi-sterile hybrid progeny. Although the reproductive behaviors of H. virescens are in many respects similar to those of H. zea (Agee, 1969), several distinct points have become apparent. Apart from the distinct pheromones of the 2 (Roelofs et al., 1974; Tumlinson et al., 1975; Klun et al., 1979), which undoubtedly impart reproductive isolation in distance communication, several other points in the sequence appear to be of importance in delivering species-specific cues.

# CHAPTER III
# EPIDERMAL GLANDS IN THE OVIPOSITOR OF *HELIOTHIS VIRESCENS* (F.)

## Introduction

Sex pheromone glands of female Lepidoptera are commonly found in the intersegmental membrane between abdominal segments 8 and 9 (Percy and Weatherston, 1974). However, cases have been reported in which the glands are found elsewhere (McFarlane and Earle, 1970; Chow et al., 1976).

As indicated in the previous chapter, studies on the reproductive behavior of *H. virescens* implicated several anatomical areas as possible sites of sex pheromone production. The present work discusses histological studies on sites of pheromone production in the terminal abdominal segments of female *H. virescens* and extends and clarifies the work of Jefferson et al. (1968).

## Methods and Materials

Female *H. virescens* were observed during the initial 3 scotophases after emergence to ensure that calling (pheromone release behavior), indicated by ovipositor extension, had developed. Actively calling females were removed from the observation cages on the 4th scotophase and placed in a freezer (-10°C) for 10 min prior to removal of the ovipositor for microscopic examination.

The terminal abdominal segments were extended by applying pressure to the abdomen, removed and pricked several times with a minuten pin to facilitate fixative infiltration prior to being placed in a dissecting

dish flooded with 2% gluteraldehyde in 0.1 M $PO_4$ buffer (pH 7.2) and a small quantity (2-3 drops) of Photoflow® to reduce surface tension. After 1 h, the material was transferred to fresh cold fixative (6°C), soaked overnight, and postfixed in 2% osmium tetroxide in 0.1 M $PO_4$ buffer (pH 7.2) for 2 h. The tissue was then dehydrated in ethanol and embedded in Spurr's resin. Both longitudinal and cross sections between 0.5 and 1.5 μm in thickness were cut with a glass knife. Sections were mounted serially on gelatin-coated slides and were stained on a hot plate (90°C) with methylene blue in 1% borax.

Ovipositors to be used in scanning electronmicroscopy (SEM) were removed using the above method and immediately placed in buffered 4% osmium tetroxide for 6 h. The tissues were dehydrated through both ethanol and ethanol-Freon® series prior to critical-point drying from Freon. They were then affixed to SEM stubs with silver conductive paint and were sputter coated with gold. SEM observations were made using a Cambridge Stereoscan MKIIA®.

## Results and Discussion

Examination of semi-thin sections revealed 2 morphologically distinct areas of glandular tissue. The most extensive area of glandular epithelia (GI in Figs. 4, 5) was situated in the intersegmental membrane (Ism) between abdominal segments 8 and 9 + 10. A 2nd area of glandular tissue (GII in Fig. 4) was found throughout the dorsal valves (papillae annales).

The glandular tissue within the Ism was found to form a chevron, similar to that of the sex pheromone producing gland of *Plodia interpunctella* (Hübner) (Smithwick and Brady, 1977a). It extended from a

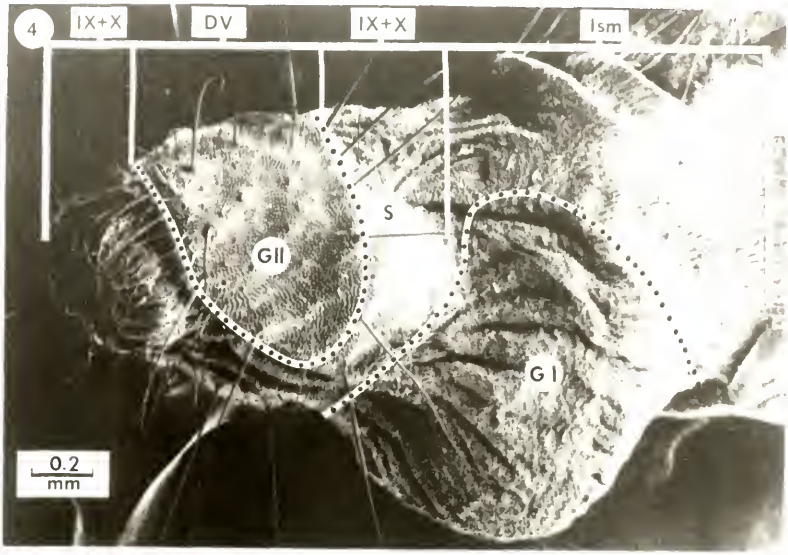
ventral position midway through the Ism to the junction of the Ism and 9th sternite and dorsolaterally to the region of the posterior apophyses (Figs. 4, 5, 6). The glandular tissue was large in surface area and showed considerable folding (Fig. 3); when exposed during calling it formed a visible bulge over the ventrolateral 2/3 of the Ism (Figs. 4, 5). Jefferson et al. (1968) found cells from this glandular area to be continuous over the dorsum, but I cannot confirm this. Epidermal cells that extended between the posterior apophyses above the hindgut were flattened, and had indistinct lateral cellular membranes. No vacuoles characteristic of pheromone gland cells were present in these epidermal cells (Fig. 7).

Cells composing the glandular tissue were similar to those found in other noctuids (Jefferson et al., 1966; Percy, 1979; Teal and Philogene, 1980). They had large central to basal nuclei and ranged from columnar over the extensive central area to cuboidal in the periphery (Figs. 6, 8). The cytoplasm was packed with vacuoles (Fig. 9). Because extracts of the Ism from both calling and noncalling females elicited male sexual responses (Chapter IV) and because similar vacuoles have commonly been associated with cells producing sex pheromones in other noctuid species (Jefferson et al., 1966; Jefferson and Rubin, 1973) it is probable that these cells are production and storage sites for at least one pheromone component.

Gland cells within the dorsal valves (GII in Fig. 4) were found to be composed of 2 distinct types (Figs. 10, 11, 12). The major portion of the glandular epithelium was composed of columnar or cuboidal cells

Figure 4. Ventrolateral aspect of the terminal abdominal segments of female H. virescens. VIII - Segment 8, IX + X - segments 9 + 10, Ism - intersegmental membrane. DV - dorsal valves, S. - setae, G.I - glandular area I, GII - glandular area 2.

Figure 5. Longitudinal section through the ovipositor. HG - hindgut; OD - oviduct; M - muscle; Ret - retractor of Gl; Ep - undifferentiated epidermal cells; VIII - abdominal segment 8; Ism - intersegmental membrane; IX + X - abdominal segments 9 + 10; DV - dorsal valves; GI - glandular area 1.


4
IX+X
DV
IX+X
lsm
S
GII
G I
0.2
mm

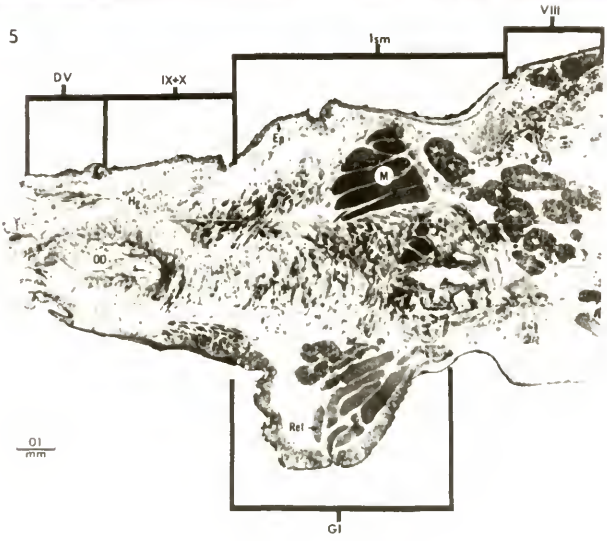
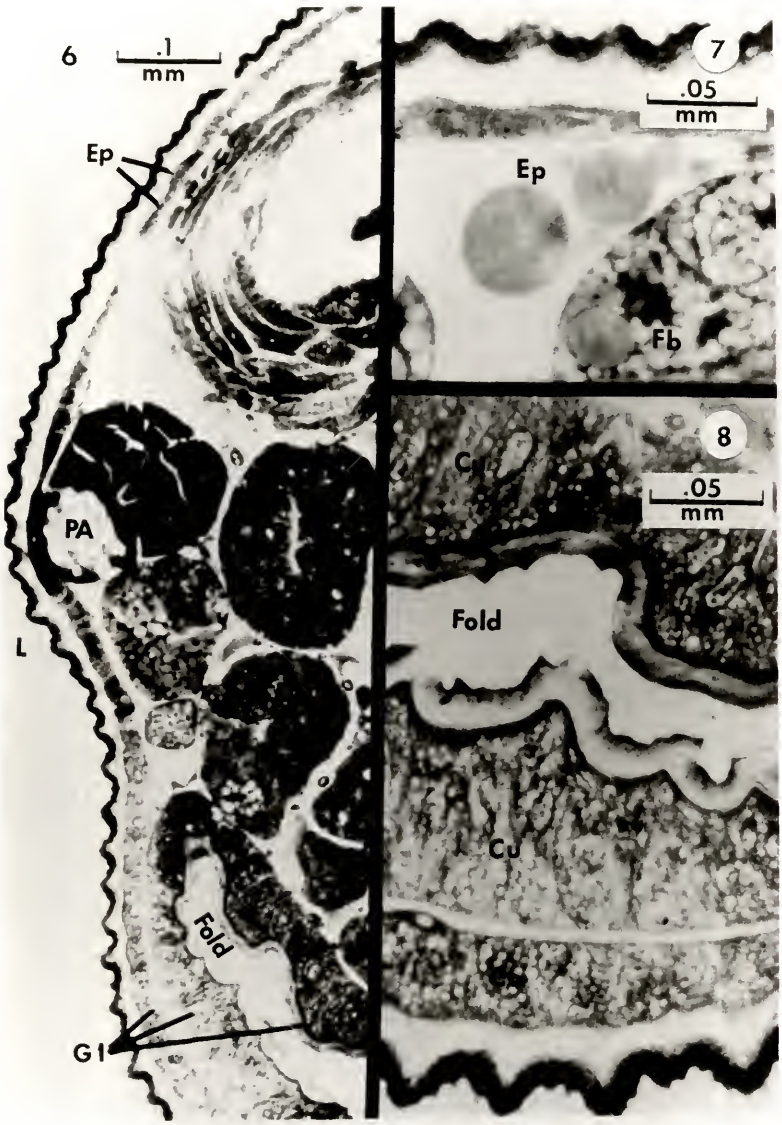

5
VIII
lsm
DV
IX+X
Ep
M
Rel
GI
01
mm

Figure 6. Cross-section through GI area of a partially extended ovipositor. PA - posterior apophysis; L - limit of GI cells, EP - undifferentiated epidermal cells. Note fold in GI.

Figure 7. Undifferentiated epidermal cells (EP) overlying the hindgut of Fig. 6. Fb - fat body.

Figure 8. Columnar (Co) and cuboidal (Cu) cells forming GI.


6
.1
mm
Ep
PA
L
Fold
G l
7
.05
mm
Ep
Fb
8
.05
mm
Cu
Fold
Cu

similar in size and structure to the gland cells found in the Ism (Fig. 11). The 2nd type of gland was composed of tormogen and trichogen cells, arranged in setiform glands and associated with tubular setae (Figs. 12, 13).

The columnar and cuboidal cells contained a considerable number of vacuoles, reminiscent of other pheromone producing glands. The dorsal valves (papillae annales) of female Lepidoptera are considered to be remnants of the terminal 11th and 12th abdominal segments (Matsuda, 1976), therefore this glandular area is considered to be distinct from that found in the 8th intersegment and adduce the differential evolution of each site. Inasmuch as the cuticle overlying pheromone producing tissue within the Ism of H. virescens and other Lepidoptera (Percy and Weatherston, 1974) has been found to be composed of loosely packed cuticle having little sclerotization, the dense, heavily sclerotized cuticle surrounding the glandular tissue of the dorsal valves was found to be quite distinct. However, it is reasonable to consider that a mechanism for pheromone release, perhaps similar to the epicuticular filaments hypothesized by Percy (1974) as being pheromone storage and release sites in Choristoneura fumiferana (Clem.), could function in this instance. Such densely packed cuticle probably precludes the cuticular storage of considerable amounts of pheromone such as the amount stored by T. ni (Percy, 1979). In fact, this cuticle may provide a mechanism for the slow release of a sex pheromone because the movement of pheromone through its heavy sclerotization may be slower than release through the intersegmental membrane. Extracts from both sites elicited

Figure 9. Columnar gland cells of GI. N - nucleus; V - vacuole; Ib - apparent infolds of apical cell membrane.

Figure 10. Longitudinal section through the dorsal valves indicating 2 glandular types (TI, TII). Hg - hindgut.

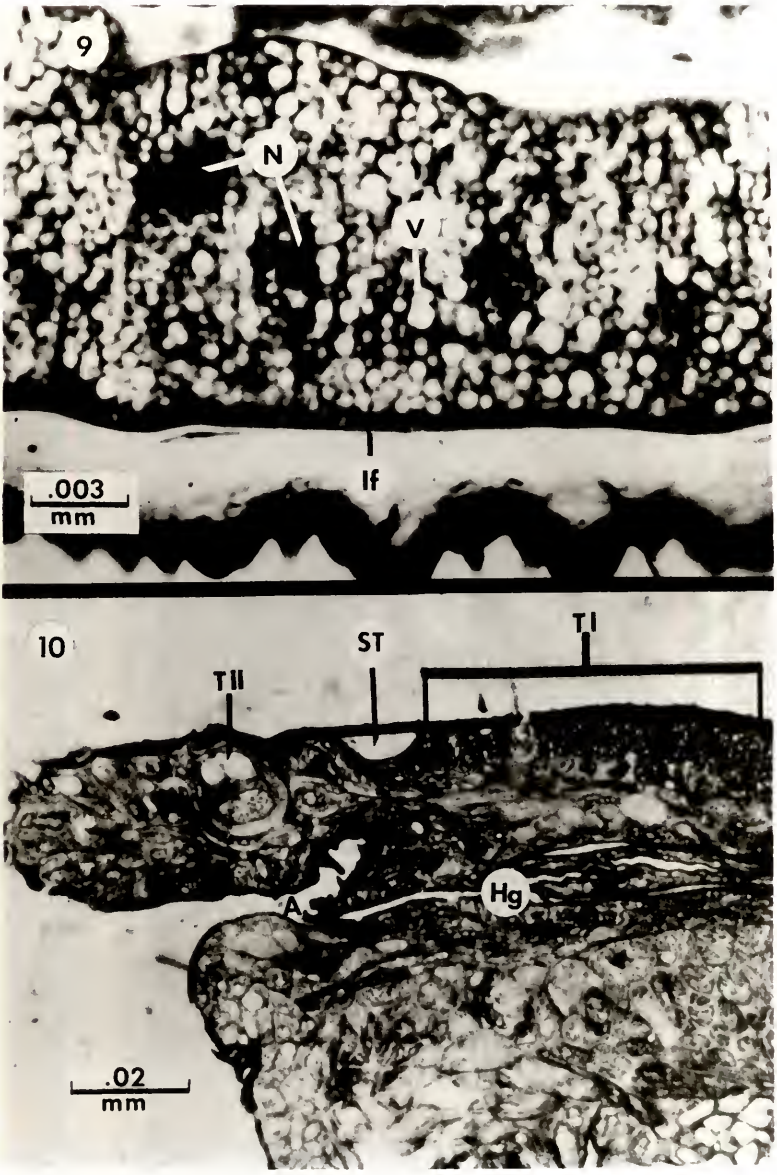
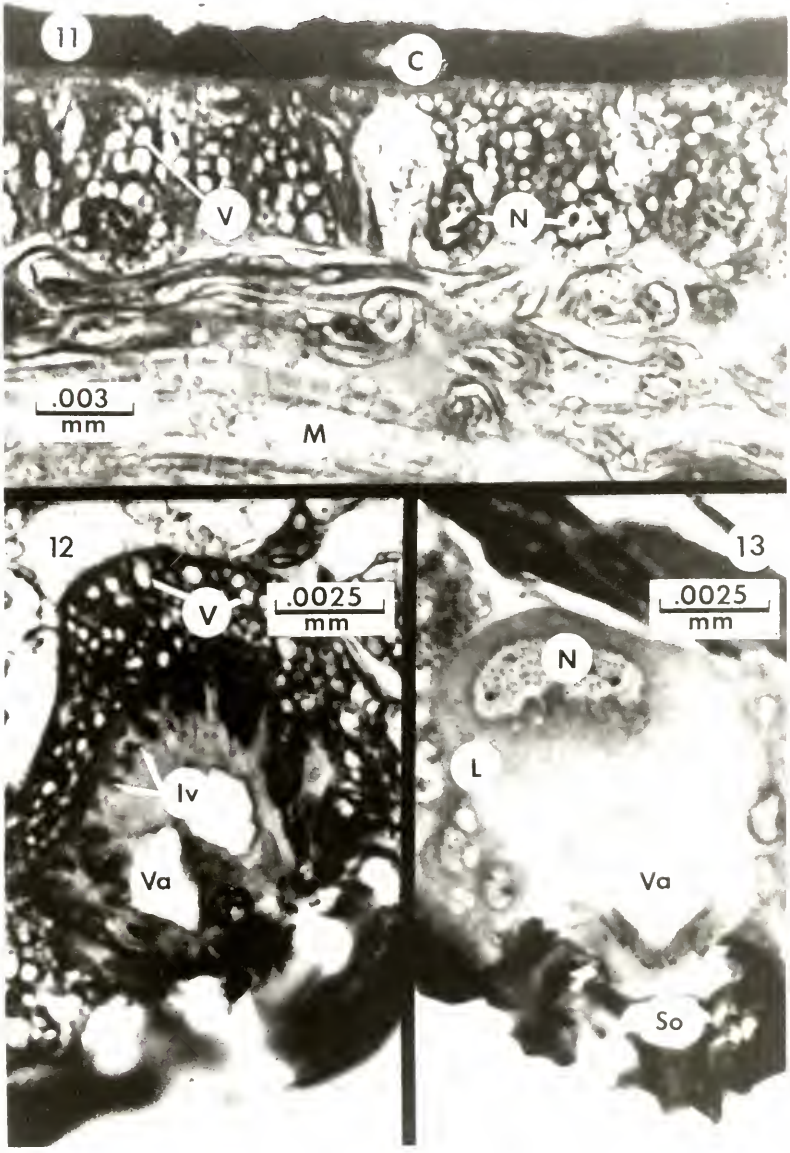

9
N
V
If
.003
mm
10
TII
ST
TI
A
Hg
.02
mm

Figure 11. Section through columnar epidermal cells of the dorsal valves. Note the heavily sclerotized cuticle (C). N - nuclei, V - vacuoles, M - muscle.

Figure 12. Section through trichogenous (TII) gland of dorsal valves. Va - large vacuole, Iv - involuted apical cell membrane, V - vacuole.

Figure 13. Section through trichogenous gland. N - lobulate nucleus, Va - large vacuole, L - lipid droplet, So - socket.


11
C
V
N
.003
mm
M
12
V
.0025
mm
Iv
Va
13
.0025
mm
N
L
Va
So

a somewhat different series of male behavioral responses in bioassays (Chapter IV) it is speculated that a slower release rate from the dorsal valves or the production of a different blend of components from that produced in the Ism may establish the precise blend of pheromone volatiles necessary for maximum effect during sexual signaling.

The trichogenous glands were far less numerous than the columnar-cuboidal gland cells and were distributed over the outer surfaces of the dorsal valves. The basal area of the trichogen cell contained a large lobulate nucleus and a considerable number of vacuoles. Apically there was a less dense area, reminiscent of the infolded apical membranes associated with other such glands, in particular male hairpencil glands (Noirot and Quennedey, 1974; Wasserthal and Wasserthal, 1977). Also, within the apical area of the trichogen cells there were variable numbers of large vacuoles (Figs. 12, 13), similar in shape and position to the lipid droplets found in the papillae annales of Euxoa species (Teal and Philogene, 1980). Although the vacuoles were apparently empty, certain sections showed traces of a slightly osmiophylic substance within the vacuoles. The contents of these large vacuoles were probably extracted during the dehydration sequence. Unfortunately, I do not known whether the contents of these vacuoles contribute to the pheromone blend released from this site. However, based on the large content of these vacuoles and the minute quantities of components obtained in gas chromatographic analyses of dorsal valve extracts I hypothesize that these secretions serve that some other function.

Although nerves occur near the basal areas of the trichogen cells, it is not known if synaptic junctions with the trichogen cells or

dendritic connectives to the tubular setae are present. However, because cases of intragland nervation are infrequent among insect species (Noirot and Quennedey, 1974), it may be that closely associated setae provide a sensory input.

# CHAPTER IV
# BEHAVIORAL AND ELECTROPHYSIOLOGICAL ASSAY OF EXTRACTS FROM TWO SEX PHEROMONE GLAND SITES OF FEMALE *HELIOTHIS VIRESCENS* (F.)

## Introduction

The pheromone blend released by female *H. virescens* was assumed to be produced by a single area of hypertrophied epidermal gland cells that form a complete ring in the 8th abdominal intersegment (Jefferson et al., 1968). However, in the previous chapter, 2 distinct areas of glandular epiderm were described in the terminal abdominal segments. The distinct morphological positions of the sites and structural differences in the cuticle overlying each area suggested that each site developed independently, perhaps contributing different component ratios to the total pheromone blend. The present study was undertaken to assess the effects of extracts from each site in the elicitation of neural responses and male sexual behaviors using the electroantennogram (EAG) technique and wind tunnel bioassay procedures.

## Methods and Materials

### Extraction of Glandular Sites

Ovipositors were removed from females during the 2nd - 7th scotoperiods following emergence. The cut end of the ovipositor was placed on absorbent paper to remove haemolymph (Klun et al., 1980a) and the respective glandular sites [8th intersegment (Ism) and posterior 2/3 of the dorsal valves (DV)] were dissected from the remaining tissue. Ethyl

ether (Mallinckrodt®, anhydrous reagent grade) extracts of whole ovipositors, and both glandular sites were stored at ca. -50°C until use.

EAG Assays

Insects used in EAG studies were immobilized in modeling clay so that the upper part of the head and antennae were free but capable of little movement. Antennal responses, recorded between the distal tip of the antennae and cranial vertex, were made using glass capillary Ag-AgCl electrodes containing 3M NaCl. Impulses were amplified by a Grass® P-16 preamplifier and monitored using a strip-chart recorder and a digital voltmeter. The sample delivery system was similar to that used by Roelofs and Comeau (1971). Compressed air, delivered at a constant rate of 200 ml/min, was used as the carrier gas and the volatiles were dispensed into the airstream through a 4-cm long glass tube (6-mm ID) containing sample-impregnated 1 X 3-cm filter papers. The output of the delivery system was positioned ca. 1 cm from the distal 2/3 of the antenna. Test samples included 0.32 female equivalent (FE), 1.00 FE, 3.20 FE, and 10.00 FE concentrations of the tissue extracts, a 1 µg standard of (Z)-11-hexadecenal, and an ether blank. All test series were begun with a blank and ended with a standard, and a 30-sec recovery period was allowed after each response. Responses were corrected and standardized by the method of Baker and Roelofs (1976).

Wind Tunnel Bioassays

A 1.5 X 0.5 X 0.5-m wind tunnel was employed to assess the relative abilities of each test extract and the recombined Ism and DV extracts to

elicit flight, tactic behavior from a distance, and close-range reproductive behaviors (Chapter II). Air speed through the tunnel was regulated by a variable speed fan so that fumes emanating from a 1 X 3-cm cotton wick impregnated with $TiCl_4$ positioned in the upwind end formed a defined plume. All tests were conducted during the peak period of male reproductive activity (Tingle et al., 1978). One x 3-cm filter papers impregnated with 1 or 10 FE concentrations of each test extract, the recombined Ism + DV extracts, or an ether blank were suspended centrally in the upwind end and individual resting males were lowered into the plume 10 cm from the downwind end. The behaviors exhibited by each of the 20 individuals tested for each sample during a 5-min test period were recorded by the observer on audio cassette tapes and later transcribed.

Frequencies of the observed behaviors were tabulated in 1st order transition matrices and ethograms were devised for each of the test samples. Chi-square values and standard normal deviate values of all transitions were calculated by common techniques (Stevenson and Poole, 1976) and were used to assess the differences in the behavioral patterns resulting from different test extracts and concentrations. In addition, populational means for each test extract were compared using Duncan's multiple range for the following criteria: the number of taxes, time prior to flight, time prior to entering the pheromone plume, time spent in the plume during the 1st orientation, time spent searching on the dispenser after the 1st landing, and the total time that males were active (i.e., vigorous wing fanning, ambulation, or flight).

## Results and Discussion

### EAG Studies

EAG responses indicated that male H. virescens perceived volatiles extracted from both the DV and Ism sites. However, responses to the DV extracts were lower than those to whole ovipositor extracts (Fig. 14). Interestingly, all concentrations of the whole ovipositor extract and higher concentrations of each gland site elicited responses of equal or greater magnitude than those obtained with the 1 µg (Z)-11-hexadecenal standard. At the 1 FE concentration the total amount of pheromone presented in a whole ovipositor sample represents 40X less than that of the 1 µg (Z)-11-hexadecenal standard (based on data of Tumlinson et al., 1975; Klun et al., 1979). Unfortunately, it is not possible to ascribe this increased neuronal output to different receptor sites on a single or several neurons without single cell analysis. However, the responses elicited from a group of 10 females by all test odorants were not significantly different from those of the blank; therefore, it is believed that the responses to these extracts are sex specific.

### Flight Tunnel Assays

Release of individual males in the flight tunnel not only provided data on the ability of test extracts to elicit male activation and upwind taxis, but also enabled the sequential analysis of an individual's behavior. As indicated in Figs. 15 and 16 the complete range of precourtship behaviors exhibited by males during mating studies (Chapter II) including: male activation, random flight, searching for the

Figure 14. Mean EAG responses of 10 males to glandular extracts. Vertical bars indicate standard errors of the mean. Responses are expressed as corrected values (test response (mV) X 10/response (mV) to 1 µg (Z)-11-hexadecenal). Stippled area represents the standardized blank response. Dashed line represents standard response to 1 µg of (Z)-11-hexadecenal.

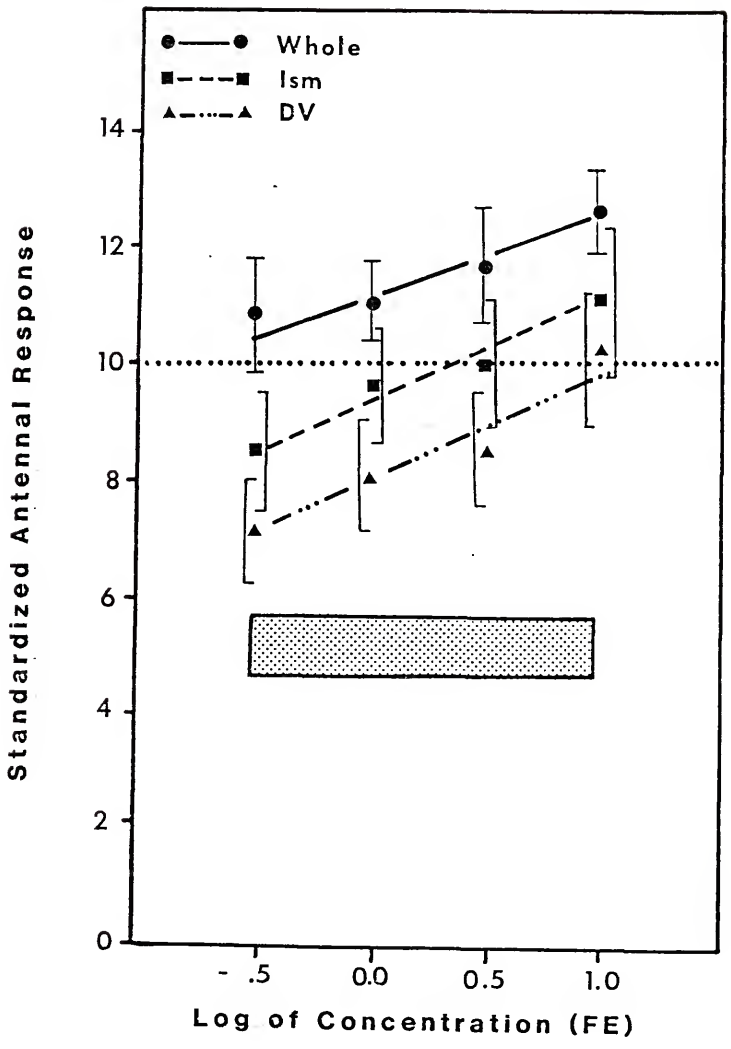

Whole
Ism
DV
Standardized Antennal Response
14
12
10
8
6
4
2
0
- .5
0.0
0.5
1.0
Log of Concentration (FE)

Figure 15. Behavioral sequences evoked by 1 FE concentration of each extract and the solvent blank in the flight tunnel. Probabilities of the individual transitions indicated are the results of monitoring 20 individually released males.

## 1 FE Samples

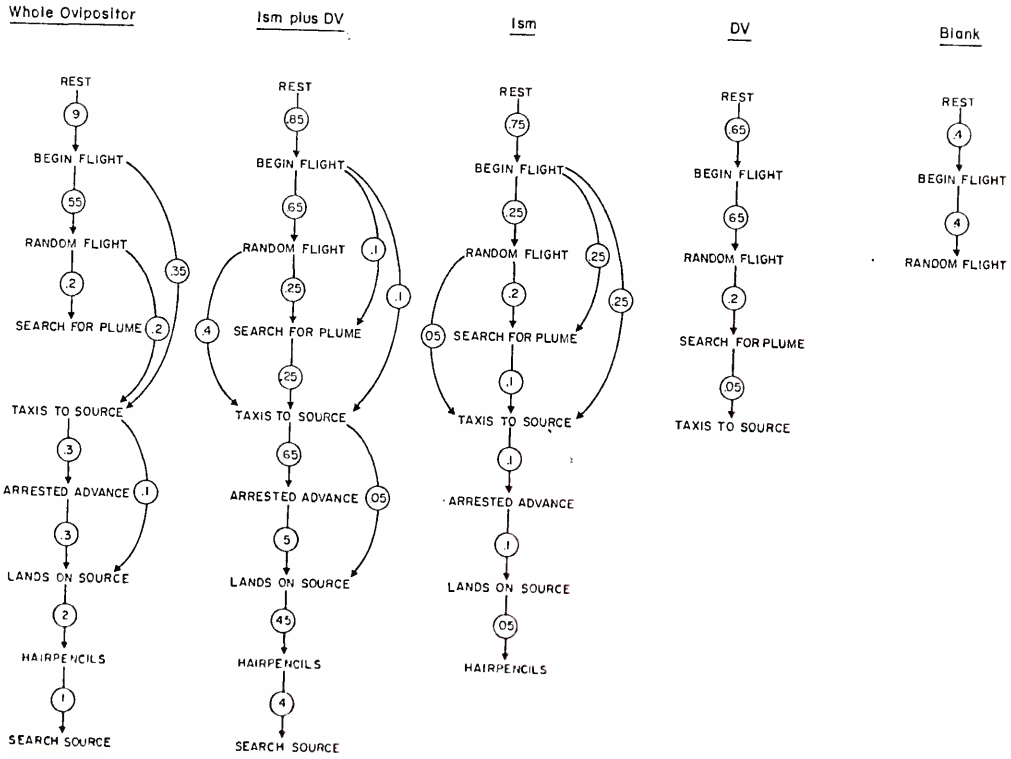

Whole Ovipositor
REST
.9
BEGIN FLIGHT
.55
RANDOM FLIGHT
.2
SEARCH FOR PLUME
.2
.35
TAXIS TO SOURCE
.3
ARRESTED ADVANCE
.1
.3
LANDS ON SOURCE
.2
HAIRPENCILS
.1
SEARCH SOURCE
Ism plus DV
REST
.85
BEGIN FLIGHT
.65
RANDOM FLIGHT
.25
.1
.4
SEARCH FOR PLUME
.25
.1
TAXIS TO SOURCE
.65
ARRESTED ADVANCE
.05
.5
LANDS ON SOURCE
.45
HAIRPENCILS
.4
SEARCH SOURCE
Ism
REST
.75
BEGIN FLIGHT
.25
RANDOM FLIGHT
.25
.2
.05
SEARCH FOR PLUME
.25
.1
TAXIS TO SOURCE
.1
ARRESTED ADVANCE
.1
LANDS ON SOURCE
.05
HAIRPENCILS
DV
REST
.65
BEGIN FLIGHT
.65
RANDOM FLIGHT
.2
SEARCH FOR PLUME
.05
TAXIS TO SOURCE
Blank
REST
.4
BEGIN FLIGHT
.4
RANDOM FLIGHT

Figure 16. Behavioral sequences evoked by 10 FE concentrations of each extract and the solvent blank in the flight tunnel. Probabilities indicated are the results of monitoring 20 individually released males.

## 10 FE Samples

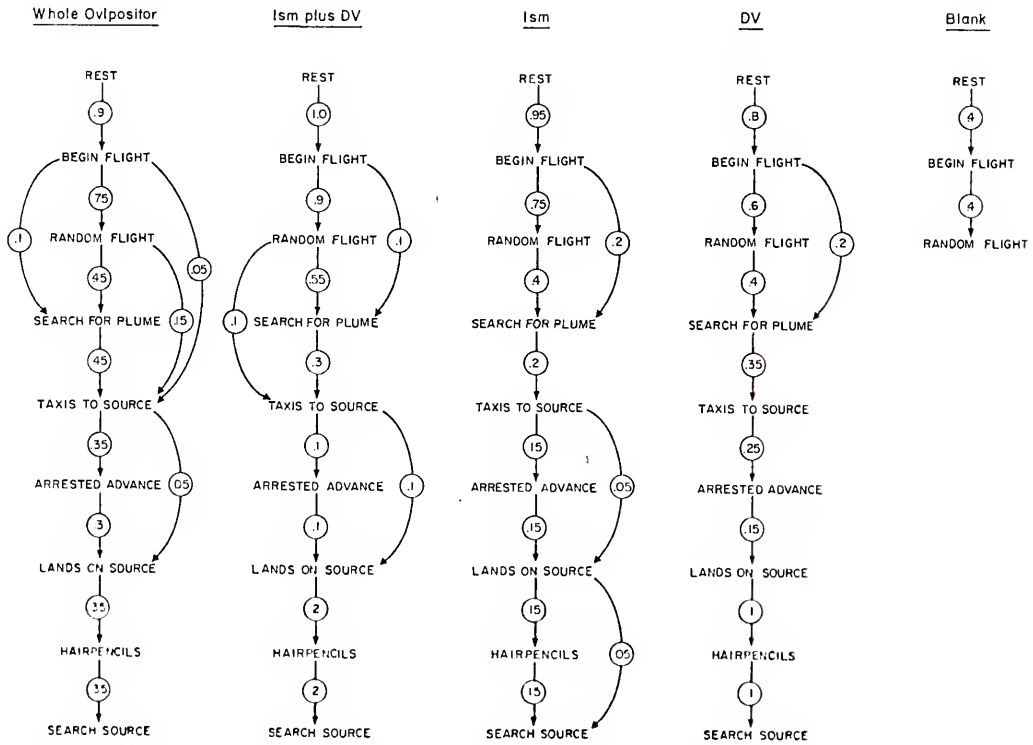


Figure 17. Male hovering at ca. 3 cm from dispenser. Note antennae are pointed toward the dispenser.

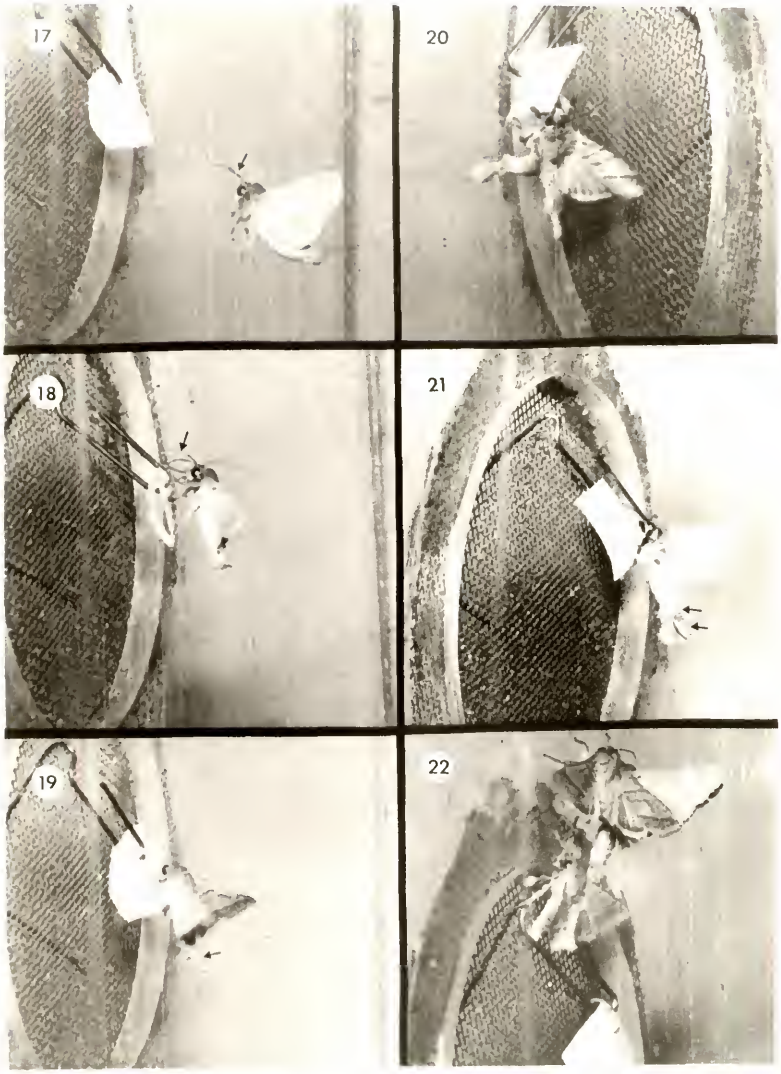
Figure 18. Same male after landing on dispenser. Note antennae (arrow).

Figure 19. Male exposing his hairpencil (arrow) on dispenser.

Figure 20. Male searching dispenser.

Figure 21. Ventral view of male searching dispenser. Note extent of hairpencil exposure (arrows).

Figure 22. Homosexual mating attempt near dispenser.


17
20
18
21
19
22

odorant plume, taxis up the plume, and a period of arrested forward advance at 5-10 cm from the odor source were observed in these assays. However, the only behaviors paralleling those exhibited during close-range male-female courtship interactions were landing on the dispenser, hairpencil exposure, and searching the dispenser (Figs. 17-22). Consequently, the effects of the test extracts in evoking such prerequisite courtship behaviors as movement under the female wing and clasping (Chapter II) remain obscure.

Extracts from each glandular site were effective in evoking more numerous responses at both concentrations than was the blank (Tables 3, 4), and behavioral transitions attributable to pheromonal cues beginning with an aerial search for the odor plume and taxis up the plume occurred only when glandular extracts were tested (Figs. 15, 16). While all of the sample extracts were equally effective in inducing males to complete the reproductive behavioral sequence assessed at the 10 FE concentration (Table 4), neither glandular site alone was capable of eliciting the complete behavioral array when 1 FE concentrations were presented (Table 3). However, 1 FE concentrations of both the whole ovipositor and recombined Ism + DV extracts were effective in inducing the complete array of behavioral events (Fig. 15). In fact, the recombined sites induced a greater behavioral array than did the whole ovipositor extract (Table 3). The difference between the whole ovipositor and Ism + DV samples can be accounted for by an increase in the number of males entering random flight and making the transitions from flight commencement or random flight to searching for the plume and

Table 3.--$\chi^2$ Comparisons of behaviors elicited by 1 FE concentrations of the test samples.

| | Samples compared[1] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Behavior | Ism + DV vs. whole ovipositor | Whole ovipositor vs. Ism | Whole ovipositor vs. DV | Whole ovipositor vs. blank | Ism + DV vs. Ism | Ism + DV vs. DV | Ism vs. DV | DV vs. blank |
| Begin flight | 0.0588 | 0.5000 | 1.3888 | 5.5550 | 0.2352 | 0.9411 | 0.2660 | 1.9230 |
| Random flight | 0.3076 | 3.2727 | 0.0909 | 0.8181 | 4.9230 | 0.0769 | 0.0769 | 1.9230[a] |
| Search for plume | 1.2857 | 6.2500 | 0.0000 | 1.0000[a] | 0.5714 | 1.2857 | 2.7777 | 4.0000 |
| Taxis to source | 1.0666 | 0.3636 | 9.0909[b] | 11.0000 | 2.4000 | 13.0666[b] | 7.1111[b] | 1.0000 |
| Arrested advance | 3.7692 | 2.6666 | 6.0000 | 6.0000 | 9.3076 | 13.0000 | 2.0000 | ------ |
| Land on dispenser | 2.5714 | 4.5000 | 8.0000 | 8.0000 | 10.2857 | 14.0000 | 2.0000 | ------ |
| Hairpencil exposure | 4.4545 | 2.2500[c] | 4.0000 | 4.0000 | 9.0909[c] | 11.0000 | 1.0000 | ------ |
| Search dispenser | 4.5000 | 2.0000 | 2.0000 | 2.0000 | 8.0000 | 8.0000 | ------ | ------ |
| $\chi^2$ | 18.0141* | 21.8030** | 30.5707** | 38.3737** | 61.3704** | 24.9555** | 15.2317* | 8.8460* |

[1]Values for each behavior are contributions to the total $\chi^2$, for each behavior $\chi^2 \geq 3.841$ is significant at 0.05

*Significant differences at 0.05.

**Significant differences at 0.01.

[a]Point after which blank elicits no further responses.

[b]Point after which DV elicits no further responses.

[c]Point after which Ism elicits no further response.

Table 4.--$\chi^2$ Comparisons of behaviors elicited by 10 FE concentrations of the test samples.[1,2]

| Behavior | Ism + DV vs. whole ovipositor | Whole ovipositor vs. Ism | Whole ovipositor vs. DV | Ism + DV vs. Ism | Ism + DV vs. DV | Ism vs. DV |
|---|---|---|---|---|---|---|
| Begin flight | 0.2222 | 0.0555 | 0.2222 | 0.0500 | 0.8000 | 0.4736 |
| Random flight | 0.6000 | 0.0000 | 0.6000 | 0.5000 | 2.0000 | 0.6000 |
| Search for plume | 0.3636 | 2.2727 | 0.909 | 0.6923 | 0.0769 | 1.0000 |
| Taxis to source | 1.9230 | 4.9230 | 2.7692 | 1.1250 | 0.1250 | 0.8000 |
| Arrested advance | 3.5714 | 2.2857 | 0.5714 | 0.5000 | 4.5000 | 1.3333 |
| Land on dispenser | 1.2857 | 1.2857 | 2.2857 | 0.0000 | 0.2500 | 0.2500 |
| Hairpencil exposure | 1.2857 | 2.2857 | 3.5714 | 0.2500 | 1.0000 | 0.3333 |
| Search dispenser | 1.2857 | 1.2857 | 3.5714 | 0.0000 | 1.0000 | 1.0000 |
| $\chi^2$ | 10.5375 | 14.3942* | 13.6823 | 3.1173 | 9.7519 | 5.7903 |

[1]Values for each behavior are contributions to the total $\chi^2$.

[2]Comparisons with the blank sample are omitted from the table because of the obvious differences between the blank and other samples (see Table 3).

*Significant difference at 0.05.

continuing the behavioral sequence from these points (Fig. 16). Although these transitions are performed by males responding to calling females the inability of males to enter taxis directly after activation suggests that the recombined Ism + DV extract contains a slightly imprecise blend of components. Hence, although flight is induced the males often search for further or appropriate stimuli prior to continuing and completing the behavioral sequence. As indicated in studies on other species (Roelofs, 1978), this may reflect an imprecise ratio of components perhaps resulting from more rigorous extraction and inclusion of other volatile components which may be somewhat disorienting (Weatherston and Maclean, 1974) when the glands were excised and extracted individually.

In assessing the points which accounted for the reduced behavioral repertoire observed during 1 FE tests of the individual gland sites (Table 5), it appears that the terminal points in the sequence are not elicited to any great extent when using the Ism extracts while the failure of males to undergo taxis during DV studies results in a reduced behavioral repertoire. Although the ability of DV extracts to evoke close-range behaviors remains obscure, the ability of whole ovipositor and recombined DV + Ism extracts to elicit the whole repertoire tends to suggest that volatiles from the Ism are responsible for inducing tactic behavior while DV extracts maximize the probability of completing courtship behaviors. However, considerable increases in the numbers undergoing taxis and subsequent behaviors during 10 FE tests of the DV extracts and a significant increase in the number of males performing

Table 5.--$\chi^2$ Comparisons of behaviors elicited by 1 and 10 FE extract concentrations.[1]

| Behavior | Extracts | | | |
|---|---|---|---|---|
| | Whole ovipositor | Ism + DV | Ism | DV |
| Begin flight | 0.0000 | 0.5294 | 0.8421 | 0.5625 |
| Random flight | 1.4545 | 1.9230 | 6.6666 | 0.0000 |
| Search for plume | 12.2500 | 5.1428 | 3.0625 | 5.3333 |
| Taxis to source | 0.3636 | 3.2666 | 3.2000 | 5.1428 |
| Arrested advance | 0.1666 | 9.3076 | 0.3333 | 5.0000 |
| Land on dispenser | 0.1250 | 7.1428 | 1.0000 | 3.0000 |
| Hairpencil exposure | 2.2500 | 4.4545 | 1.3333 | 2.0000 |
| Search dispenser | 12.5000 | 2.0000 | 4.0000 | 2.0000 |
| $\chi^2$ | 29.1098** | 33.7671** | 20.4379** | 23.0386** |

[1]Values for each behavior are contributions to the total $\chi^2$.

**Significant differences at 0.01.

the terminal step when 10 FE samples of the Ism were presented (Table 4), indicates that neither site is absolutely necessary for the elicitation of any step in the behavioral sequence (Baker et al., 1979).

Results obtained from the analysis of the number of orientations and means of the temporal criteria measured tend to support the hypotheses established in sequential analysis studies (Table 6). All of the gland extracts caused males to begin flight significantly earlier than the blank samples suggesting that pheromonal cues responsible for the initiation of flight are present within each gland site. Further, although the 1 FE concentration of the DV extract resulted in a significantly longer pretactic flight period than 1 FE concentrations of either the whole or Ism + DV samples, a 10X increase in the DV concentration resulted in values for all criteria which were not significantly different from those obtained using 10 FE of the whole ovipositor sample. Hence the DV gland site does appear to produce an effective pheromone blend. The number of insects used in analysis of times spent moving up the plume and searching the dispenser was variable due to the relative ability of each extract to elicit both behaviors (Figs. 15, 16). Hence, the observed results are of limited value. However, the data do suggest that males spent about the same length of time moving up the plume for all extracts evaluated. Because the probability of entering the pheromone plume is both extract and concentration dependent (Figs. 15, 16) it may be that if a male perceives sufficient stimulae to enter the plume then he will move upwind at a constant rate provided the stimulus is not removed (see Kennedy and

Table 6.--Comparison of the effects of test samples on temporal and behavioral aspects of *H. virescens* male reproductive behaviors.[1]

| | Means of criteria assessed | | | | | |
|---|---|---|---|---|---|---|
| Test sample | Total time active (sec) | Time prior to flight (sec) | Time prior to taxis up plume (sec) | Time in taxis during 1st orientation (sec) 23 | Total no. orientations 25.4 | Time spent searching dispenser 1st time (sec) |
| 10 FE whole ovipositor | 205.00$^{a,b,c}$ | 41.65$^{a,b}$ | 119.25$^{a}$ | 15.33 (12)$^{a}$ | 9.52 (7)$^{b}$ | 1.40$^{a}$ |
| 10 FE Ism + DV | 264.40$^{a}$ | 19.90$^{a}$ | 203.85$^{b}$ | 10.33 (9)$^{a}$ | 29.75 (4)$^{a}$ | 0.95$^{a,b}$ |
| 10 FE Ism | 209.15$^{a,b}$ | 20.50$^{a}$ | 183.60$^{a,b}$ | 8.40 (5)$^{a}$ | 19.33 (3)$^{a,b}$ | 0.80$^{a,b,c}$ |
| 10 FE DV | 162.65$^{b,c,d}$ | 99.45$^{b,c}$ | 169.30$^{a,b}$ | 11.78 (9)$^{a}$ | 8.00 (2)$^{b}$ | 0.55$^{b,c,d}$ |
| 1 FE whole ovipositor | 212.20$^{a,b}$ | 60.35$^{a,b,c}$ | 202.70$^{b}$ | 10.00 (7)$^{a}$ | 4.60 (5)$^{b}$ | 0.65$^{a,b,c,d}$ |
| 1 FE Ism + DV | 195.00$^{b,c}$ | 63.05$^{a,b,c}$ | 179.50$^{a,b}$ | 15.82 (11)$^{a}$ | 4.55 (9)$^{b}$ | 1.30$^{a,b}$ |
| 1 FE Ism | 141.15$^{c,d}$ | 108.25$^{b,c}$ | 222.35$^{b}$ | 19.13 (8)$^{a}$ | -------- | 0.60$^{b,c,d}$ |
| 1 FE DV | 118.05$^{d,e}$ | 121.40$^{c}$ | 294.00$^{c}$ | --------- | -------- | 0.05$^{c,d}$ |
| Blank | 76.15$^{e}$ | 209.90$^{d}$ | 300.00$^{c}$ | --------- | -------- | 0.00$^{d}$ |

[1]Means followed by the same letter are not significantly different at a 0.05 level in a Duncan's multiple range test.

[2]Numbers in ( ) indicate the number of observations used in analysis.

[3]Because no males entered taxis during blank tests a-d only 1 entered taxis when the 1 FE concentration of the DV extract was presented, neither sample was used in analysis.

[4]The blank, 1 FE of the Ism, and 1 FE of the DV extracts were eliminated from analysis because no males performed the behavior.

Marsh, 1974). However, significantly less time was spent in an active state when 1 FE of either of the individual gland sites were presented than when 1 FE of the whole ovipositor extract was used. Further the whole ovipositor and Ism + DV extracts were always at least as effective as either of the individual gland sites. Therefore, it would appear that, here again, volatiles from both glandular areas act in concert to minimize the time necessary for males to respond to and find sexually responsive females.

In summary, results of the EAG, sequential behavioral analysis, and temporal analysis studies all strongly support the hypothesis that discrete epidermal glands in both the 8th intersegmental membrane (Ism) and dorsal valves (DV) produce a volatile sex pheromone blend. Further, both gland sites apparently produce volatiles which act in concert to maximize the probability that a male will effectively seek a sexually active female and enter into courtship with her. This optimization of the pheromone blend has been shown to be of critical importance to several species (Baker et al., 1976; Carde et al., 1977), and has been hypothesized as being imperative for the maintenance of reproductive isolation between some species (Baker and Carde, 1979). The development of the sex pheromone gland within the dorsal valves of H. virescens may have been a response to direct reproductive competition between it and both H. zea (Boddie) and H. subflexa (Gn.) because all 3 have distinctly similar sex pheromones, sympatric ranges, and intersecting mating periods (Tingle et al., 1978; Klun et al., 1980b; Chapter VII). Such a development would, perhaps, not only finely tune the pheromone blend of

H. virescens, thereby providing highly effective male behavioral releasers, but could also impart a measure of semiochemical isolation through the production of a blend which was unattractive to other species. Although direct support for this hypothesis is unavailable, critical behavioral evaluations of volatiles emitted from anatomical areas which have been shown to have marginal sex pheromone activity in other species, particularly those species living in sympatricity with close relatives (Chow et al., 1976; Smithwick and Brady, 1977b) may help to support or refute this hypothesis.

Possibility of Inter-gland Contamination

A distinct problem in conducting this study was the very close proximity of the individual gland sites to one another. Therefore, although care was taken to remove only the posterior 2/3 of the DV there was a distinct possibility that pheromone components originating within the Ism might contaminate the surface of the DV and give rise to dubious results. However, gas chromatographic analysis of DV extracts using both 50 m OV-101® and 66 m SP-2340® capillary columns indicate the presence of a (Z)-9-tetradecenal:(Z)-11-hexadecenal ratio of ca. 4:1. Ratios reported for these 2 compounds by Tumlinson et al. (1975) and Klun et al. (1980b) were 1:16 and 1:25.4 [(Z)-9-tetradecenal: (Z)-11-hexadecenal], respectively. Hence, the ratio of these 2 compounds in the DV extracts is reversed indicating that contamination was probably not a factor in these biological studies.

# CHAPTER V
# THE FUNCTION OF SEX PHEROMONES IN THE REPRODUCTIVE ISOLATION OF *HELIOTHIS VIRESCENS* (F.) FROM *HELIOTHIS SUBFLEXA* (GN.) UNDER LABORATORY CONDITIONS

## Introduction

Recently, considerable emphasis has been placed on the control of the tobacco budworm, *Heliothis virescens* (F.), by genetic means. The basis of this approach lies in the production of sterile male and fertile female hybrid and backcross progeny resulting from matings between *H. virescens* males and females of a related species, *H. subflexa* (Gn.). However, such laboratory matings occur very infrequently and commonly result in the production of inviable offspring (Brazzel et al., 1953; Laster, 1972). Interspecific matings between *H. subflexa* males and *H. virescens* females are even more difficult to obtain although the resultant hybrids appear to suffer fewer genetic abnormalities than the aforementioned hybrid group (Proshold and LaChance, 1974). Hence, it would appear that this pair of species have developed effective mechanisms of both pre- and postmating reproductive isolation.

Among vagile, related species sharing common ranges, reproductive isolation is commonly effected by mechanisms acting prior to copulation (Mayr, 1970), and although several such mechanisms may be involved, disparate sex pheromone blends appear to be of major importance for many species of Lepidoptera (Roelofs and Carde, 1974; Carde et al., 1977) including *H. virescens* and *H. zea* (Boddie) (Klun et al., 1979). Judging from the sympatric distributions and overlapping mating periods reported for *H. subflexa* and *H. virescens* (Tingle et al., 1978) a similar

situation may be responsible for their genetic isolation. However, differences in the ease of obtaining the 2 hybrid stocks indicate that males of each species may respond quite differently to the pheromone produced by females of the other species. The following work discusses the results of tests designed to assess the influence of semiochemicals on reproductive isolation between H. subflexa and H. virescens.

## Methods and Materials

Heliothis subflexa were reared from eggs obtained from 1st to 3rd generation laboratory stocks maintained at Gainesville, FL. Adults were maintained as described in Chapter II, and held in isolation from members of both the opposite sex and different species.

All bioassays were conducted in a 1.5 X 0.5 X 0.5-m wind tunnel and during the period of overlapping mating activity described by Tingle et al. (1978). In tests designed to assess the ability of actively calling H. virescens females to elicit male sexual responses from male H. subflexa, groups of 3-5 females were placed on a tobacco plant in the upwind end of the flight tunnel during the prereproductive period. Similarly, a Physallis plant provided a calling site for groups of 3-5 H. subflexa females during both intra- and interspecific mating studies. Visual (Shorey and Gaston, 1970) and tactile cues (see Chapter II) have been implicated as behavioral releasors for male reproductive behaviors among several lepidopterous species so tests were also conducted using ethyl ether gland extracts obtained from actively calling females of each species. In each of these tests a 1 X 3-cm filter paper impregnated with a 1 FE (female equivalent) concentration of the test extract

was suspended centrally in the upwind end of the flight tunnel. Males used in all tests were released individually into the plume 1.4 m downwind from the semiochemical source. The behavioral reactions of each of the 20 males released for each assay were observed and recorded on audio-cassette tapes by the observer during the 5-min test period. Males that were active prior to being lowered into the plume were not considered for analysis.

Frequencies of the observed behaviors were tabulated in 1st-order transition matrices prior to formulating ethograms. Expected values of each transition were calculated and compared using common techniques (Chapter II).

## Results and Discussion

### Intraspecific Heliothis subflexa Mating

Although many aspects of both the male and female reproductive behaviors of H. subflexa are similar to those performed by H. virescens differences do exist.

The most prominant difference between the reproductive behaviors of male H. subflexa and H. virescens is partial genital segment exposure by H. subflexa males prior to and during tactic flight ($p = 0.5$). However, male H. subflexa are capable of mating with both H. virescens (Proshold and LaChance, 1974) and H. subflexa females without genital exposure during precourtship behaviors. Therefore, although this behavior is distinct and provides a good indication that males are sexually stimulated it is impossible to infer that it will lead to further reproductive behaviors or that a particular blend of synthetic pheromone

components which elicit this behavior will provide the stimuli necessary for the completion of mating (Baker and Carde, 1979a).

Female H. subflexa are generally more passive than are H. virescens during the protracted period of calling and can, in some instances, be manually repositioned while calling. This passive behavior may explain the absence from the genitalia of male H. subflexa of the long hairpencils present on the genitalia of both male H. virescens and H. zea. This group of hairpencils appears to function in the dissemination of a female arrestant during H. virescens mating and since H. subflexa females seldom attempt escape when courted by H. subflexa ($p = 0.05$) males, the evolution of these structures by both H. virescens and H. zea males appears to be a species-specific event. Further, because 56% of the interspecific courtships between H. virescens males and H. subflexa females were terminated by female escape (Fig. 25), the development of a species-specific arrestant pheromone by both H. virescens and H. zea seems probable. Such species-specific "aphrodisiac" pheromones are thought to function in the behavioral isolation of P. interpunctella and Cadra cautella (Walker) (Grant et al., 1974) and in both cases appear to provide a final premating mechanism of isolation.

## Behavioral Interactions between Heliothis virescens and Heliothis subflexa

Studies on the semiochemically induced interactions and behaviors between H. subflexa and H. virescens indicated that males of the 2 species respond quite differently to the naturally released sex pheromone of the other species.

Thirteen of the 20 male H. subflexa released into the pheromone plume created by calling H. virescens females exhibited activation behaviors including wing fanning, ambulation, and genital exposure (Fig. 23). However, the mean time for these behaviors to occur was 3.37 min (as opposed to ca. 1 min in conspecific studies), indicating that although some semiochemical signal was being perceived it was not of sufficient quality to induce an immediate behavioral response. Further, of those males which committed activation behaviors, fewer were induced to take flight, the only other behavior occurring with regularity (binomial test, $p = <0.05$). Results of the study employing 1 FE of the H. virescens pheromone bland extract were in general agreement with the above results ($\chi^2$, 6dF). However, there was a slight decrease in the number of male H. subflexa becoming active and no males either entered taxis or committed an aerial search in the upwind end of the tunnel (Fig. 24).

Certain reproductive behaviors can be elicited using single pheromone components or even synthetic chemicals having some similarity to known components (Roelofs, 1978), so it seems probable that pheromone components shared by the 2 species are responsible for the elicitation of the above responses. However, since only a single male was successful in finding the H. virescens pheromone plume and entering taxis, and because no males were successful in finding females when upwind, cues necessary for the maximization and completion of reproductive behaviors by H. subflexa males are absent from the naturally produced H. virescens pheromone blend.

Figure 23. Behaviors performed by initially inactive H. subflexa males released downwind from actively calling H. virescens females. Values indicate the probabilities that a specific behavioral transition will occur.

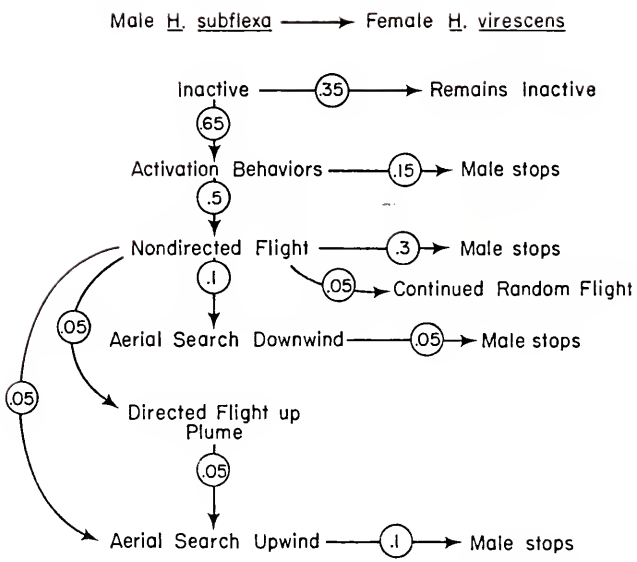
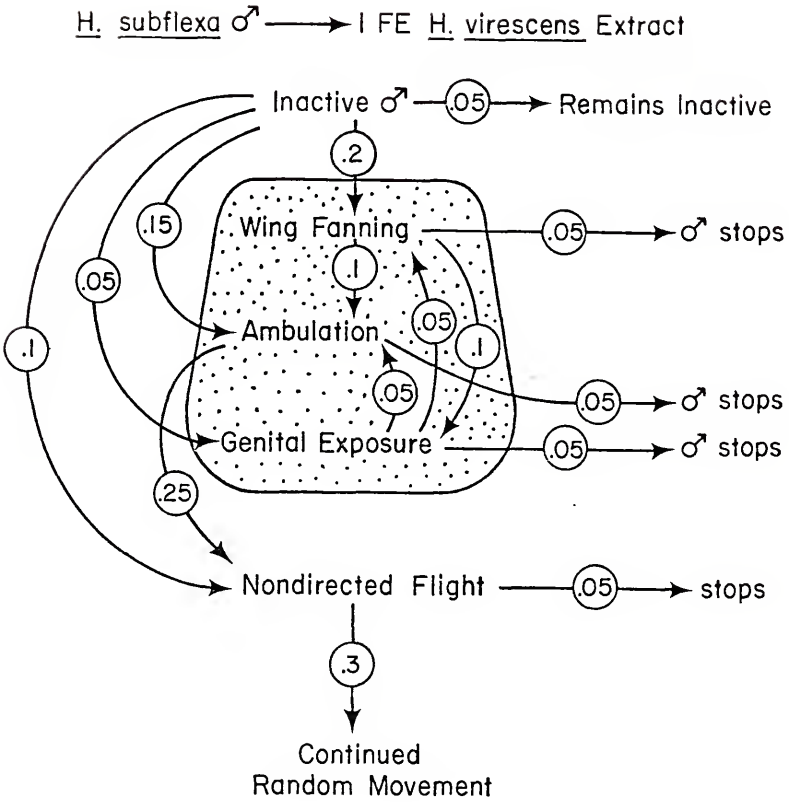

Male H. subflexa → Female H. virescens
Inactive
.35
Remains Inactive
.65
Activation Behaviors
.15
Male stops
.5
Nondirected Flight
.3
Male stops
.1
.05
Continued Random Flight
.05
Aerial Search Downwind
.05
Male stops
.05
Directed Flight up Plume
.05
Aerial Search Upwind
.1
Male stops

Figure 24. Behaviors performed by initially inactive H. subflexa males released downwind from a 1 FE sample of the H. virescens pheromone gland extract. Values indicate the probability that a specific behavioral transition will occur. Stippled area includes activation behaviors.


H. subflexa ♂ → 1 FE H. virescens Extract
Inactive ♂
.05
Remains Inactive
.2
.15
Wing Fanning
.05
♂ stops
.05
.1
.1
Ambulation
.05
.05
.1
.05
♂ stops
Genital Exposure
.05
♂ stops
.25
Nondirected Flight
.05
stops
.3
Continued
Random Movement

The most effective blend of pheromone components identified from H. subflexa female gland extracts has 2 components in common with H. virescens, (Z)-9-hexadecenal and (Z)-11-hexadecenal, and 3 unique acetates, (Z)-7-hexadecen-1-ol acetate, (Z)-9-hexadecen-1-ol acetate, and (Z)-11-hexadecen-1-ol acetate (Chapter VI). Preliminary field studies indicate that at the very least, the 2 $C_{16}$ aldehydes plus (Z)-11-hexadecen-1-ol acetate are of importance to the capture of male H. subflexa. Hence, the absence of these 3 acetate components from the H. virescens pheromone (see Klun et al., 1979) and different ratios of (Z)-9-hexadecenal:(Z)-11-hexadecenal probably account for the semiochemical isolation between H. subflexa males and H. virescens females. However, the release of pheromone components by female H. virescens such as tetradecanal, (Z)-9-tetradecenal, and (Z)-11-hexadecanol which may be disorienting to male H. subflexa and cannot be discarded as a possible mechanism for reproductive isolation.

The behavioral repertoire exhibited by male H. virescens in response to the pheromone blend produced by calling H. subflexa females was quite distinct from that described above (Fig. 25). Only 10% of the initially inactive male H. virescens failed to fly during these tests and in fact, the probability of undergoing taxis toward calling H. subflexa females ($p = 0.80$) was not significantly different from that found in conspecific mating studies (Chapter II). Therefore, chemical cues responsible for the elicitation of flight and tactic behavior by male H. virescens are released by calling H. subflexa females. However, while only 7% of the males failed to land, or approach, calling H.

virescens females in conspecific mating studies (Chapter II), 25% were incapable of completing these behaviors in response to calling H. subflexa females. This reduction in the number of males making contact with females obviously contributes substantially to the very low probability of interspecific mating ($p = 0.15$) ($p = 0.56$, conspecific H. virescens matings). Hence, it appears that semiochemically imparted reproductive isolation between H. virescens males and H. subflexa females results from the release of a pheromone blend that does not provide a stimulus of sufficient magnitude to induce a high percentage of the males to land and subsequently enter into courtship. Chemically, this may result from the distinct ratio of (Z)-9-hexadecenal:(Z)-11-hexadecenal produced by female H. subflexa (Chapter VII), and the probable absence of both tetradecanal and (Z)-9-tetradecenal from the H. subflexa blend. However, because a number of males do perform courtship behaviors after contacting the female, disparities between the pheromone blends of H. virescens and H. subflexa are not solely responsible for reproductive isolation. Rather, it seems that both the pheromone blend and the females' ability to escape from courting males ($p = 0.25$) function as major inputs to reproductive isolation.

Cross-attraction between H. subflexa females and H. virescens males has been shown to occur under field conditions (Tingle et al., 1978), although the number of males captured was much lower than the number landing near calling H. subflexa females in our studies. This reduction in cross-attraction under natural conditions was expected because species usually maintain premating mechanisms of reproductive isolation

more effectively in the wild (Dobzhansky, 1951; Smith, 1953), and under no-choice situations such as those used in this study, other closely related noctuids have been shown to overcome barriers to reproductive isolation (Byers and Hinks, 1978). Hence, it would appear that differences in pheromone composition are the major factors contributing to reproductive isolation under natural conditions and that female escape behavior functions in a backup capacity.

Results of experiments employing 1 FE of the H. subflexa sex pheromone gland extract were considerably different from tests in which calling females were used (Fig. 26). In fact the range of behaviors performed was so reduced that behaviors involved in the male activation phase were assessed individually. This indicates that the pheromone blend released from the H. subflexa extract was quite different from that released by calling females. This is not an uncommon feature encountered when using pheromone gland extracts and, in fact, the blend of chemicals identified from H. subflexa gland extracts contained a concentration of at least 1 mono-unsaturated $C_{16}$ alcohol which was a conspecific landing inhibitor (Chapter VI). The disparity between the blends may involve a very slight difference in the component ratios (Baker and Cardé, 1979a), but it effectively shuts down long-distance semiochemical communication when presented at a 1 FE concentration level. Interestingly, small cage bioassays using the small chamber described in Chapter II indicate that males do perceive sufficient chemical stimulae from a 1 FE sample to perform such close-range courtship behaviors as searching the dispenser and hairpencil exposure.

Figure 25. Behaviors performed by initially inactive H. virescens males released downwind from actively calling H. subflexa females. Values indicate the probability that a specific behavioral transition will occur.

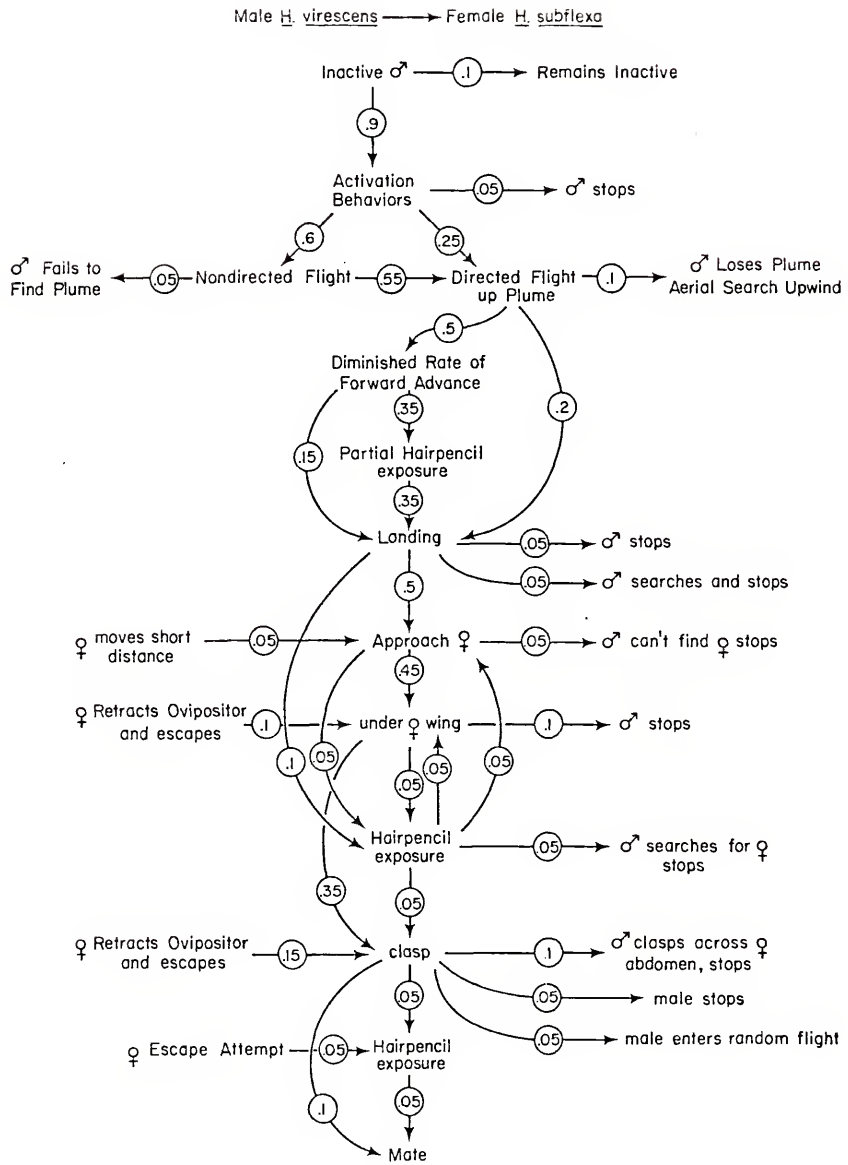
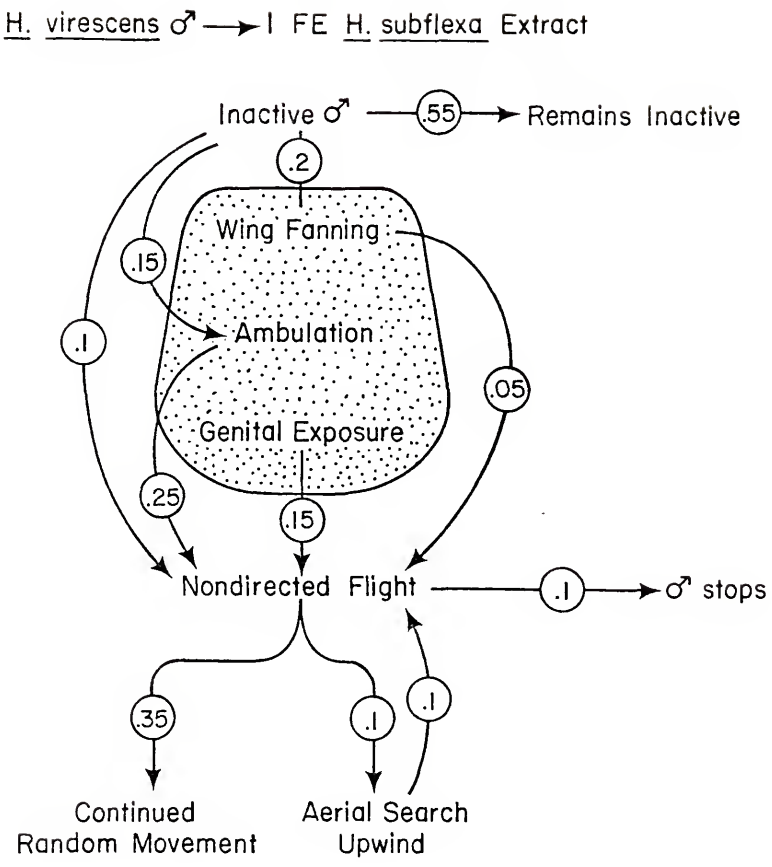

Male H. virescens → Female H. subflexa
Inactive ♂
.1
Remains Inactive
.9
Activation Behaviors
.05
♂ stops
.6
.25
♂ Fails to Find Plume
.05
Nondirected Flight
.55
Directed Flight up Plume
.1
♂ Loses Plume Aerial Search Upwind
.5
Diminished Rate of Forward Advance
.35
.2
.15
Partial Hairpencil exposure
.35
Landing
.05
♂ stops
.05
♂ searches and stops
.5
♀ moves short distance
.05
Approach ♀
.05
♂ can't find ♀ stops
.45
♀ Retracts Ovipositor and escapes
.1
under ♀ wing
.1
♂ stops
.1
.05
.05
.05
.05
Hairpencil exposure
.05
♂ searches for ♀ stops
.35
.05
♀ Retracts Ovipositor and escapes
.15
clasp
.1
♂ clasps across ♀ abdomen, stops
.05
.05
male stops
.05
male enters random flight
♀ Escape Attempt
.05
Hairpencil exposure
.05
.1
Mate

Figure 26. Behaviors performed by initially inactive H. virescens males released downwind from a 1 FE sample of the H. subflexa pheromone gland extract. Values indicate the probability that a specific behavioral transition will occur. Stippled area includes activation behaviors.


H. virescens ♂ → 1 FE H. subflexa Extract
Inactive ♂
.55
Remains Inactive
.2
Wing Fanning
.15
Ambulation
.1
.05
Genital Exposure
.25
.15
Nondirected Flight
.1
♂ stops
.35
.1
.1
Continued Random Movement
Aerial Search Upwind

Hence, it would appear that a relatively specific blend of components is necessary for the induction of tactic behavior by male H. virescens, and that while this blend may act to maximize the probability that a male will enter courtship (see Baker and Carde, 1979a), it is not an absolute prerequisite.

Effects of Non-Pheromonal Cues on Male Behavior

Although vision and perhaps sound (see Dahm et al., 1971) cannot be negated as possible cues for the induction of male taxis, the ability of the H. subflexa extract to elicit courtship behaviors from H. virescens males when tested in a small active space indicates that neither of these factors play major roles in the elicitation of male reproductive behaviors.

Recent preliminary field studies (McLaughlin and Huettel, unpublished) indicate that the attraction of male H. virescens to calling H. virescens females does not depend strongly if at all upon the host crop. Further, because H. subflexa males reacted similarly to both calling H. virescens females plus a tobacco plant and H. virescens gland extracts in the absence of the H. virescens host plant, it would appear that volatiles from the tobacco plant exerted little behavioral influence over H. subflexa. Hence, although no tests have, as yet, been conducted in which females were used in the absence of their respective host plants, the potential influence of host plant volatiles on male reproductive behavior is not considered to be very great. However, the precise influences of host plant volatiles on both male and female reproductive behavior cannot be critically defined without further extensive study.

# CHAPTER VI
# IDENTIFICATION OF A SEX PHEROMONE OF HELIOTHIS SUBFLEXA (GN.) AND FIELD TRAPPING STUDIES USING DIFFERENT BLENDS OF COMPONENTS

## Introduction

Recent chemical studies on several Heliothis species have indicated the existence of a common trend in the types of compounds forming the pheromone blend, with (Z)-11-hexadecenal and (Z)-9-hexadecenal being components common to all species studied thus far (Klun et al., 1980a,b; Nesbitt et al., 1979, 1980). Hence, it appears that pheromonal mechanisms of isolation between species result from different blends and ratios of components. This chapter reports the identification of a highly effective species-specific sex pheromone blend produced by female H. subflexa.

## Methods and Materials

### General

Pheromone extracts were prepared from groups of 5 actively calling H. subflexa females which had entered into a protracted bout of calling as indicated by ovipositor extension for a minimum of 2 min. Individual calling females were removed from the holding cage and the ovipositors removed as described by Klun et al. (1980a). Immediately after removal the ovipositors were placed, 5 per micro-vial, in 250 µl of ethyl ether (Mallinckrodt®, anhydrous reagent grade) and allowed to soak for a period of 2 to 3 min. The ethyl ether extract was pipetted into another vial which contained 15 µl of isooctane (Fisher, 99 Mole %) and the

ether allowed to evaporate under a fine nitrogen stream. Extracts were then stored at -60°C until use.

Chemical Analysis

Gas chromatographic (GC) analysis of 3 to 5 µl samples (1-2 female equivalents (FE)) of the ovipositor extracts was done with a Hewlett-Packard Model 5710A® GC equipped with a splitless injector system. The output of the flame ionization detector was interfaced to a Nicolett 1180® data system capable of storing 32K real time data points. Nitrogen (linear flow velocity of 9.8 cm/sec) was used as a carrier gas. Initial GC studies were performed with a 66 m X 0.25 mm (ID) glass capillary column coated with SP2340 (Supelco, Belefonte, PA) capable of separating most of the geometrical and positional isomers of $C_{14}$ and $C_{16}$ alcohols, aldehydes and acetates (Heath et al., 1980). Samples were injected at an initial column temperature of 60°C (injector temperature = 250°C) with a 60 sec delay prior to injector purging. The column temperature was programmed after 2 min at 32°C/min to a final temperature of 150°C. Extracts were also chromatographed on a 31 m X 0.25 mm (ID) glass column coated with cholesteryl cinnamate (Heath et al., 1979). Splitless injections were made at the mesophase transition temperature (159°C) using decane as the solvent.

The retention times of the compounds eluting during GC analysis of the ovipositor extracts as well as those of the synthetic standards were reduced to equivalent chain length units (ECL) with slight modification (Jamison and Reid, 1969). For the purpose of this study, the acetates of primary saturated alcohols varying in chain length from 12 to 20

carbons were used as the functional retention index (Swoboda, 1962), regardless of the compound's functionality. In subsequent studies, ovipositor extracts were co-chromatographed with isomerically pure (98%+) synthetic standards.

Further chemical characterization was accomplished by GC-mass spectrometry with a Finigan Model 3200® chemical ionization mass spectrometer equipped with a GC inlet. The combined extract from 50 calling females was injected onto a 2 m X 2 mm (ID) glass column packed with 3% OV-17® on Gas Chrom Q® (100/120 mesh) and the total effluent was introduced directly into the ionization source. Methane was used as both a carrier and reagent gas. Spectra of the natural products were compared with those of candidate synthetic compounds.

All synthetic standards used (Fig. 27) with the exception of (E)-7-hexadecenal, (E)-9-hexadecenal, (E)-11-hexadecenal, (E)-9-hexadecen-1-ol, (E)-11-hexadecen-1-ol, and (Z)-9-hexadecen-1-ol acetate were obtained from Chemical Samples Co. (Columbus, OH), as were the starting materials used in preparation of the above standards. (E)-9-Hexadecen-1-ol was obtained from a lithium bronze reduction of 9-hexadecyn-1-ol (Mueller and Gillick, 1978) and a portion of the product was oxidized to (E)-9-hexadecenal with pyridinium chlorochromate (Corey and Suggs, 1975). (E)-7-Hexadecenal and (E)-11-hexadecenal were similarly prepared by oxidation of their corresponding alcohols. The (E)-11-hexadecen-1-ol was obtained by saponification of (E)-11-hexadecen-1-ol acetate while the (Z)-9-hexadecen-1-ol acetate was prepared by acetylation of (Z)-9-hexadecen-1-ol. Isomeric purity was assessed by GC analysis with the SP2340 column and, when necessary, geometric isomers were separated by high performance liquid chromatography on a 25 cm X 2.5 cm (OD) $AgNO_3$

column eluted with toluene (Heath and Sonnet, 1980). All standards were assessed as being at least 98% pure.

Bioassays and Field Testing

Laboratory-reared males were used to assess the ability of gland extracts to elicit male reproductive behaviors. In these tests, 1 FE samples of the extracts were placed on 1 X 3 cm filter papers and suspended in the upwind end of a 1.5 X 0.5 X 0.5 m plexiglass wind tunnel. Individual males were then released into the center of the downwind end and behaviors monitored over a 5 min period. The data were recorded on audio-cassette tape and later transcribed. The ability of each extract to induce upwind taxis, landing on the dispenser, and genital segment exposure was assessed.

Field studies were conducted during July and August of 1980 in a fallow field near Gainesville, FL, containing ground cherry, Physalis sp., which supported a high larval H. subflexa population. Cone traps (Hartstack et al., 1979) spaced 10 m apart were set in 2 lines at 90° to one another in the field. The traps were randomly baited with 3 calling-age females, a 2 ml polyethylene vial containing 30 mg of the synthetic mixture plus 5 µg of BHT (2,6-bis(1,1-dimethylethyl)-4-methylphenol) as an antioxidant, or a blank vial. Both the females and vials were rebaited every 2 days. In a 2nd series of tests traps were baited with 3 females, a blank 8.5-cm diam. filter paper, or filter papers baited at 30-min intervals during the calling period with either 5 FE of the ovipositor extract or an equivalent amount (ca. 75 ng as indicated

by GC analysis) of the synthetic blend. All baits were re-randomized daily.

The species specificity of the H. subflexa synthetic blend was assessed during field trapping studies in tobacco and corn fields having populations of either H. virescens or H. zea (Boddie) and in the original test field. All sites had fruiting ground cherry present. Cone traps, spaced from 10 to 12 m apart and located in the vicinity of Physalis plants, were baited with 3 female H. subflexa, 30 mg of the H. subflexa synthetics dispensed from polyethylene vials, 3 female H. zea or H. virescens, or 30 mg of either the 4-component H. zea or the 7-component H. virescens synthetic blend reported by Klun et al. (1980a,b) depending upon the field. Tests in the original test field included females and synthetic blends of all 3 species.

Two series of tests were conducted to assess the effects of groups of components on trap capture. In the 1st series the effect of deleting the alcohol, acetate, or aldehyde components from the blend was assessed with rubber septa (A. H. Thomas Co.) (Flint et al., 1979) impregnated with the same concentration of blend components as in the 25 mg complete blend. The 2nd test was designed to assess the effectiveness of the aldehyde, alcohol, and acetate component groups independently, and to assess combinations of the aldehyde and acetate fractions. Pherocon® 1C sticky traps spaced 10 m apart were positioned in 2 lines in the original field and randomized daily during the test periods. Daily trap captures were recorded and the data transformed to $\log_{10}(n + 1)$ prior to statistical analysis.

## Results and Discussion

Components contained within the ovipositor extracts were tentatively identified by comparison of their ECL values with those of a standard mixture of synthetic compounds. This mixture contained the Δ7, Δ9, and Δ11 isomers of hexadecenal, hexadecen-1-ol, and hexadecen-1-ol acetate. The saturated analogs of the above compounds were also added to the mixture. Although the SP2340 column provided adequate resolution of most of the compounds (Fig. 27), the cholesteryl cinnamate column was also used to further define the assignments of the components. Table 7 lists the ECL units for the synthetic compounds used in this study on both the SP2340 and cholesteryl cinnamate columns.

Bioassays of 1 FE concentrations of the whole ovipositor extracts gave consistent results indicating that the complete range of reproductive behaviors assessed was elicited with glandular extracts while only minimal random flight was observed when solvent blanks were presented.

Capillary chromatography of these extracts revealed several major peaks having retention times coinciding with hexadecanal, (Z)-9-hexadecenal, (Z)-11-hexadecenal, (Z)-7-hexadecen-1-ol acetate, (Z)-9-hexadecen-1-ol acetate, (Z)-11-hexadecen-1-ol acetate, (Z)-9-hexadecen-1-ol, and (Z)-11-hexadecen-1-ol (Figs. 27-30). Several other peaks were also variably present but, when present, each composed less than 1% of the total mixture.

Mass spectra obtained from peaks eluting from the OV-17 column when ovipositor extracts were injected had identical fragmentation patterns

Table 7.--Equivalent chain length units (ECL) of GC standards on the SP2340 and cholesteryl cinnamate capillary columns as calculated using saturated $C_{14}$-$C_{16}$ acetates as the functional retention index.

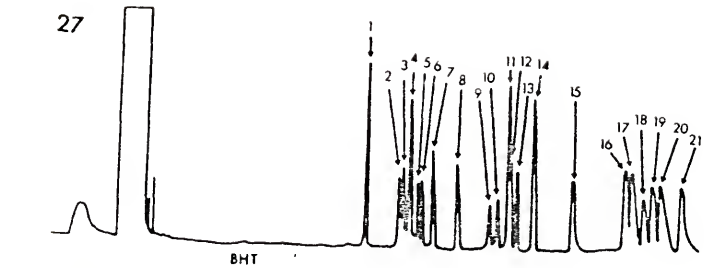
| | Equivalent chain length | |
|---|---|---|
| Standard | SP2340 | Cholesteryl cinnamate |
| Hexadecanal | 1513 | 1434 |
| (E)-7-Hexadecenal | 1555 | 1412 |
| (Z)-7-Hexadecenal | 1577 | 1402 |
| (E)-9-Hexadecenal | 1559 | 1415 |
| (Z)-9-Hexadecenal | 1582 | 1404 |
| (E)-11-Hexadecenal | 1567 | 1419 |
| (Z)-11-Hexadecenal | 1584 | 1410 |
| Hexadecen-1-ol acetate | 1600 | 1600 |
| (E)-7-Hexadecen-1-ol acetate | 1628 | 1578 |
| (Z)-7-Hexadecen-1-ol acetate | 1645 | 1566 |
| (E)-9-Hexadecen-1-ol acetate | 1634 | 1583 |
| (Z)-9-Hexadecen-1-ol acetate | 1653 | 1571 |
| (E)-11-Hexadecen-1-ol acetate | 1643 | 1585 |
| (Z)-11-Hexadecen-1-ol acetate | 1665 | 1575 |
| Hexadecan-1-ol | 1693 | 1561 |
| (E)-7-Hexadecen-1-ol | 1734 | 1521 |
| (Z)-7-Hexadecen-1-ol | 1752 | 1508 |
| (E)-9-Hexadecen-1-ol | 1737 | 1526 |
| (Z)-9-Hexadecen-1-ol | 1756 | 1511 |
| (E)-11-Hexadecen-1-ol | 1744 | 1541 |
| (Z)-11-Hexadecen-1-ol | 1768 | 1518 |

Figure 27. Chromatogram of standard compounds eluting from the SP2340 column. 1 = Hexadecanal, 2 = (E)-7-hexadecenal, 3 = (E)-9-hexadecenal, 4 = (E)-11-hexadecenal, 5 = (Z)-7-hexadecenal, 6 = (Z)-9-hexadecenal, 7 = (Z)-11-hexadecenal, 8 = hexadecan-1-ol acetate, 9 = (E)-7-hexadecen-1-ol acetate, 10 = (E)-9-hexadecen-1-ol acetate, 11 = (E)-11-hexadecen-1-ol acetate, 12 = (Z)-7-hexadecen-1-ol acetate, 13 = (Z)-9-hexadecen-1-ol acetate, 14 = (Z)-11-hexadecen-1-ol acetate, 15 = hexadecan-1-ol, 16 = (E)-7-hexadecen-1-ol, 17 = (E)-9-hexadecen-1-ol, 18 = (E)-11-hexadecen-1-ol, 19 = (Z)-7-hexadecen-1-ol, 20 = (Z)-9-hexadecen-1-ol, 21 = (Z)-11-hexadecen-1-ol.

Figure 28. Chromatogram of components in the ovipositor extracts eluting from the SP2340 column. 1 = Hexadecanal, 6 = (Z)-9-hexadecenal, 7 = (Z)-11-hexadecenal, 12 = (Z)-7-hexadecen-1-ol acetate, 13 = (Z)-9-hexadecen-1-ol acetate, 14 = (Z)-11-hexadecen-1-ol acetate, 20 = (Z)-9-hexadecen-1-ol, 21 = (Z)-11-hexadecen-1-ol.

Figure 29. Chromatogram of standard compounds eluting from the cholesteryl cinnamate column. Compound labels as in Fig. 27.

Figure 30. Chromatogram of components in the ovipositor extracts eluting from the cholesteryl cinnamate column. Component labels as in Fig. 28.


27
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3
4
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17
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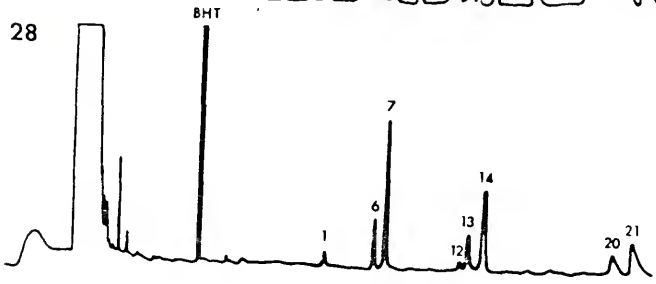
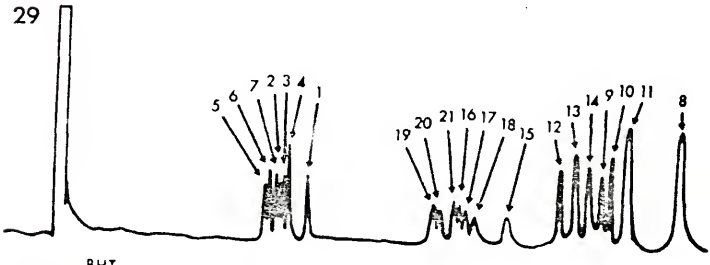
21


28
BHT
7
6
14
13
12
1
20
21


29
5
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7
2
3
4
1
19
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21
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8

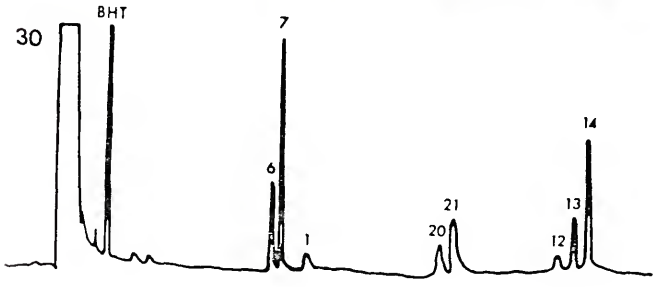

30
BHT
7
6
1
21
20
14
13
12

Table 8.--Chemical components isolated from H. subflexa ovipositor washes.

| Compound | Mean % composition (15 ♀) | Composition by weight (ng/♀) |
|---|---|---|
| Hexadecanal | 5.4 | 0.82 |
| (Z)-9-Hexadecenal | 19.8 | 3.01 |
| (Z)-11-Hexadecenal | 30.0 | 4.56 |
| (Z)-7-Hexadecen-1-ol acetate | 1.6 | 0.25 |
| (Z)-9-Hexadecen-1-ol acetate | 4.3 | 0.65 |
| (Z)-11-Hexadecen-1-ol acetate | 12.3 | 1.87 |
| (Z)-9-Hexadecen-1-ol | 14.4 | 2.19 |
| (Z)-11-Hexadecen-1-ol | 12.2 | 1.85 |

with the mono-unsaturated aldehyde, acetate, and alcohol standards. Points of unsaturation were further confirmed by co-chromatography of the ovipositor extracts and synthetic compounds on the SP2340 capillary and cholesteryl cinnamate columns. The relative proportion of each of these compounds within the natural blend is indicated in Table 8.

Field tests of the synthetic blend formulated either in polyethylene vials or on filter paper indicated that the synthetics were as effective in capturing males as either females or the crude ovipositor extracts in cone traps (Table 9). Field observations indicated that males generally underwent upwind taxis toward the traps baited with the synthetic blend, extending their genitalia and hovering at 10-15 cm from the dispenser prior to flying up and into the trap. However, close range courtship behaviors such as landing on the dispenser, abdominal curving, and copulatory attempts were not observed when the synthetic blend was used, which indicates that cues responsible for eliciting courtship behaviors were absent, masked, or inhibited in these tests. Such stimuli may result from several different features absent in our tests such as the precise blend of components released by calling females, visual, and tactile stimuli (Baker and Carde, 1979a,b). Further, the absence of stimuli responsible for landing and close-range copulatory behaviors are presumed to have little effect upon cone trap efficiency since males commonly move up and out of the pheromone plume when incomplete stimuli are presented and are therefore captured in cone traps.

Table 9.--Comparison of cone trap captures of male H. subflexa using females, synthetic chemicals, and crude extracts.[a]

| Trap bait | Mean trap capture*/night |
|---|---|
| 3 Females | 7.6 a |
| 20 mg Synthetics in vials | 8.0 a |
| 5 FE synthetics on filter papers | 7.3 a |
| 5 FE ovipositor extracts on filter papers | 3.0 a |
| Blank | 0.0 b |

[a]Means followed by the same letter are not significantly different at a 0.05 level in a Duncan's multiple range test.

*Six replicates.

Neither the synthetic blends nor caged females of the 3 species captured males of other Heliothis species, indicating that the pheromone blends are species specific. However, while both the H. subflexa and H. virescens synthetic blends caught as effectively as caged females (15 ♂ to H. subflexa synthetics/21 ♂ to H. subflexa ♀, and 6 ♂ to H. virescens synthetics/10 ♂ to H. virescens ♀) the blend described for H. zea by Klun et al. (1980a) was considerably less effective than females (3 ♂ to synthetics/56 ♂ to ♀). This may indicate a disparity in the actual blend released during calling and that maintained within the pheromone gland or cuticle overlying it (cf. Weatherston and Maclean, 1974). Although the obvious differences in the blends of the 3 species suggest several avenues by which reproductive isolation could be effected the behavioral effects of differences in the blend ratios or presence or absence of components on any of the 3 species have not been assessed, and I am unable to define the basis for chemical isolation at present. However, laboratory interspecific communication studies between H. virescens and H. subflexa indicated that long distance semiochemical isolation occurs between female H. virescens and male H. subflexa. However, it is the close-range orientation that is disrupted when the male H. virescens are released downwind from calling H. subflexa females.

Studies conducted to assess the effects of deleting the alcohol, aldehyde, or acetate components from the blend indicated that the removal of any one of these groups did not completely stop trap capture (Table 10, experiment 1). While the whole synthetic blend was as

effective as virgin females in cone trap studies, a significant decrease in captures relative to females was noted when the whole blend was employed in sticky traps. Further, considerably more males were captured in the sticky traps when the alcohols were deleted, suggesting that the alcohols may act as an inhibitor to landing, a prerequisite for both mating and being caught in sticky traps. This is further supported by field observations of moths orienting to cone traps which indicated that although males did approach dispensers containing the whole blend none landed or exhibited any close-range copulatory behaviors.

Tests employing baits composed of various blends of components in sticky traps indicated that those containing either the acetates or alcohols alone were ineffective in trapping males while the binary mixture of (Z)-9-hexadecenal and (Z)-11-hexadecenal was considerably more effective than the whole blend of alcohols, aldehydes, and acetates (Table 10, experiment 2). The acetates alone are ineffective trap baits but their addition to the binary mixture of mono-unsaturated aldehydes causes a pronounced increase in the number of males captured, indicating their necessity for effective sexual communication. The decrease in captures recorded on addition of hexadecanal to either the mono-unsaturated aldehyde or mono-unsaturated aldehyde + acetate blends tends to suggest a slight inhibitory function. However, insufficient behavioral analysis has been conducted to determine the validity of this hypothesis at present.

Although the blend reported here is highly complex and may contain chemicals having no behavioral significance, a definite biochemical

Table 10.--Comparison of sticky trap captures of H. subflexa males using different blends of components.[a,b,c,d,e]

| 3 ♀ | 16:Al | Z9-16:Al | Z11-16:Al | Z7-16:Ac | Z9-16:Ac | Z11-16:Ac | Z9-16:OH | Z11-16:OH | Mean (males/night) |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Experiment no. 1 | | | | | |
| + | | | | | | | | | 2.742 a |
| | + | + | + | + | + | + | | | 1.912 a |
| | + | + | + | | | | + | + | 0.774 b |
| | + | + | + | + | + | + | + | + | 0.706 b |
| | | | | + | + | + | + | + | 0.045 c |
| | | | | | | | | | 0.000 c |
| | | | | Experiment no. 2 | | | | | |
| | | + | + | + | + | + | | | 2.899 (a) |
| | + | + | + | + | + | + | | | 1.000 (b) |
| | | + | + | | | | | | 0.941 (b) |
| | + | + | + | | | | | | 0.803 (b,c) |
| | + | + | + | + | + | + | + | + | 0.578 (c,d) |
| | | | | + | + | + | | | 0.148 (c,d) |
| | | | | | | | + | + | 0.148 (c,d) |
| | | | | | | | | | 0.000 (d) |

Table 10.--continued.

[a]Raw data transformed to $\log_{10}$ (X+1) prior to analysis (16 replicates over 8 nights experiment 1; 10 replicates over 5 nights experiment 2).

[b]Means followed by the same letter are not significantly different in a Duncan's multiple range test at a 0.05 level.

[c]16:Al = Hexadecanal; Z9-16:Al = (Z)-9-hexadecenal; Z11-16:Al = (Z)-11-hexadecenal; Z7-16:Ac = (Z)-7-hexadecen-1-ol acetate; Z9-16:Ac = (Z)-9-hexadecen-1-ol acetate; Z11-16:Ac = (Z)-11-hexadecen-1-ol acetate; Z9-16:OH = (Z)-9-hexadecen-1-ol; Z11-16:OH = (Z)-11-hexadecen-1-ol.

[d]Means from the 2 experiments are not compared with one another.

[e]The presence of a compound in a test blend is indicated by a +.

theme based on the use of $C_{16}$ compounds is quite obvious. The use of these compounds is common among heliothids throughout the world with (Z)-11-hexadecenal being the major pheromone component in all cases reported (Klun et al., 1980a,b; Nesbitt et al., 1979, 1980). The distinction appears to be the presence of acetates within the H. subflexa blend. However, Rothschild (1978) has recently reported that a mixture of (Z)-11-hexadecenal, (Z)-9-tetradecenal, and (Z)-11-hexadecen-1-ol acetate caught considerably more male H. punctigera than did the aldehyde blend alone. Hence, while the aldehydes are of obvious major importance to sexual signaling, the acetates may indeed form an integral part of the pheromone blend of many Heliothis species.

# CHAPTER VII
# SUMMARY AND CONCLUSIONS

## Summary

1. The reproductive behavior of Heliothis virescens can be broadly categorized into precourtship and courtship behaviors. Precourtship behaviors include female calling, male activation, orientation toward the female, and male landing near the female. Courtship involves numerous variably committed behaviors by each sex but generally includes: 1) male movement under the female wing, 2) female wing fanning, 3) partial hairpencil exposure, 4) male moving parallel with the female, 5) pair turning to face each other, and 6) male clasping the females genitalia.

2. A high percentage of male H. virescens were incapable of finding and successfully mating with conspecific females during their first pheromonally induced upwind flight under laboratory conditions. However, subsequent reorientations increased the probability of successful mating substantially. Failure to successfully mate most often resulted from the male's failure to move parallel with the female prior to clasping, although a substantial number of females rejected males which were committing the appropriate behaviors. These female rejections seem to be the result of courtship encounters in which the female has not entered the quiescent calling phase.

3. Two areas of glandular tissue in the ovipositor of H. virescens, one in the 8th abdominal intersegmental membrane and the other in the dorsal valves, were identified histologically and behaviorally as being

sites of sex pheromone production. Apparently the admixture of volatiles released from each site is necessary to maximize the behavioral effects of the pheromone. Further, because extracts from each site elicited a different range of male reproductive behaviors it is assumed that each site is responsible for the production of a distinct blend of pheromone components.

4. Wind tunnel bioassays of the semiochemically induced reproductive interactions between H. virescens and H. subflexa indicated that H. subflexa males were incapable of orienting to calling H. virescens females from a distance. H. virescens males were able to orient toward calling H. subflexa females and many performed some courtship behaviors. However, the majority of encounters between H. virescens males and H. subflexa females failed to culminate in mating because of an inability of the male to find the female when at close range or to complete the required courtship sequence and because female H. subflexa often escaped from H. virescens males when courted. Hence, pheromonally induced reproductive isolation between H. subflexa males and H. virescens females occurs at a distance while close-range behavioral isolation functions in the isolation of male H. virescens and female H. subflexa under no-choice laboratory conditions.

5. The sex pheromone gland of calling H. subflexa females was found to contain 8 compounds which were identified as hexadecanal, (Z)-9-hexadecenal, (Z)-11-hexadecenal, (Z)-7-hexadecen-1-ol acetate, (Z)-9-hexadecen-1-ol acetate, (Z)-11-hexadecen-1-ol acetate, (Z)-9-hexadecenol, and (Z)-11-hexadecenol. Field testing using cone traps baited

with as little as 5 FE of the 8-component blend indicated that it was a highly effective male attractant. However, field observations, indicating that males failed to land on the pheromone dispenser, coupled with very reduced male trap captures recorded when stick traps were employed, suggested that the gland-extract blend contained at least one male landing inhibitor. This hypothesis was supported in subsequent tests in which groups of components were eliminated from the pheromone blend. Results of these studies indicated that the best blend for attracting and inducing male H. subflexa to land was a 5-component blend made up of 29.1% (Z)-9-hexadecenal, 44.1% (Z)-11-hexadecenal, 2.4% (Z)-7-hexadecen-1-ol acetate, 6.3% (Z)-9-hexadecen-1-ol acetate, and 18.1% (Z)-11-hexadecen-1-ol acetate. Further, the incorporation of the 2 mono-unsaturated alcohols into the blend distinctly reduced sticky trap captures indicating that at least one may function as a landing inhibitor.

## Conclusions

The heliothid moths are a group with worldwide distribution and can be divided into two genera, Heliothis and Helicoverpa (Hardwick, 1965). These are distributed throughout the New and Old World. Although sex pheromones and attractants have been categorized for only five species, the presence of (Z)-9-hexadecenal and (Z)-11-hexadecenal in all of these suggests the development of a $C_{16}$ aldehyde-based communication system early in tbe evolution of the group. It is therefore possible to use the taxonomic and chemical data accumulated to arrive at some hypotheses concerning the evolution of this group of species. The most primitive of the Helicoverpa is H. punctigera,

an Australian endemic. On that continent it is sympatric with H. armigera. The latter species, however, is distributed over almost the entire eastern hemisphere. It is generally more abundant and has a wider host range in Australia than H. punctigera. Therefore, it is likely that the incorporation of $C_{16}$ mono-unsaturated acetates into the pheromone blend of H. punctigera females (Rothschild, 1978) resulted from selection pressure to preclude the attraction of the more numerous H. armigera males and, thereby, interspecific matings.

Helicoverpa zea and H. armigera appear to be more closely related but occupy an allopatric distribution, the latter being restricted to the New World. In this instance, interspecific premating isolation breaks down readily in the laboratory (Hardwick, 1965) and the pheromone blends have been shown to be nearly identical (Klun et al., 1979; Nesbitt et al., 1980), both being composed primarily of the $C_{16}$ aledhydes. However, since the two species have become sympatric on some Pacific islands, it is reasonable to assume that reproductive isolation will be reinforced in those isolated localities through sexual selection.

The premating reproductive isolation of H. zea, H. virescens and H. subflexa is more clearly understood. All three are seasonally synchronic and sympatric over large portions of their distributions. Therefore, the use of (Z)-11-hexadecenal and (Z)-9-hexadecenal as pheromone components by all three species suggests that without the evolution of more refined pheromone blends considerable interspecific attraction would occur (Tingle et al., 1978; Teal and Byers, 1980). If the H. zea-H. armigera blend may be considered primitive for purposes of this discussion, then we find that

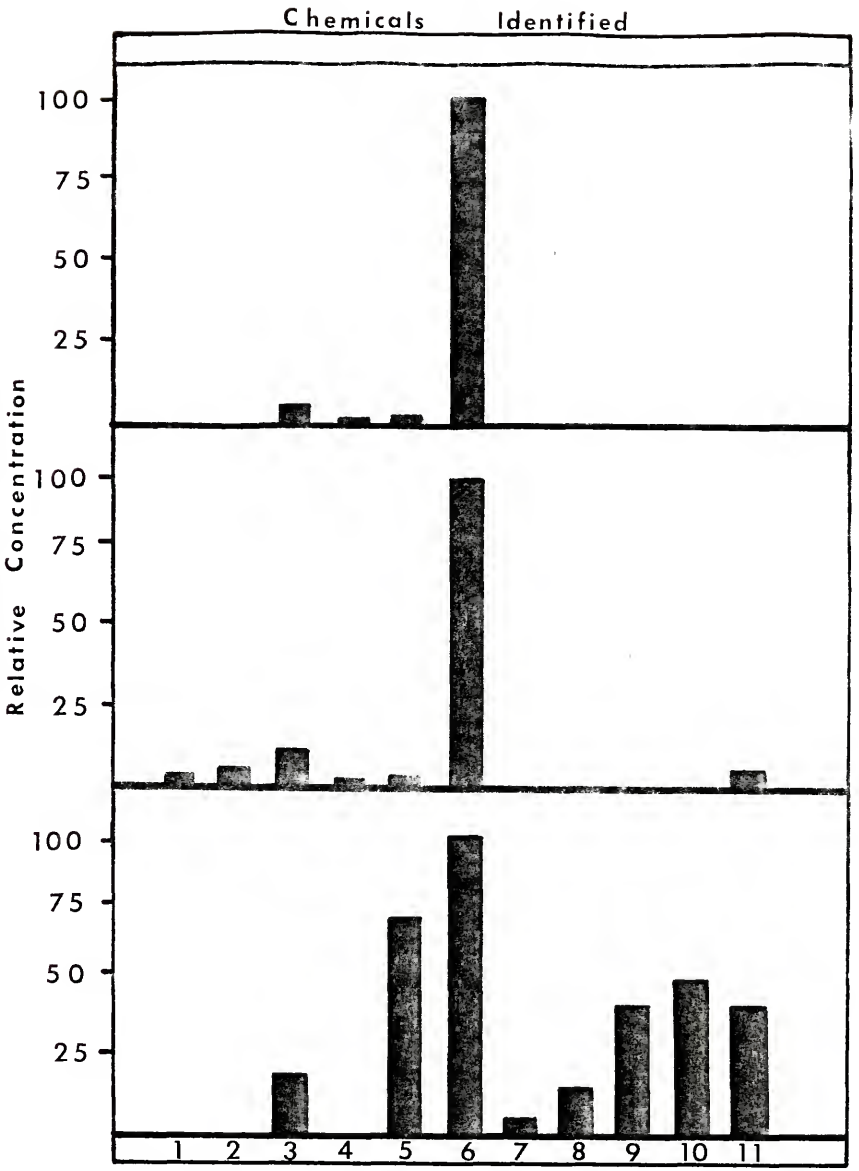
the Heliothis species have diverged from it by the evolution of additional components. Heliothis virescens maintains approximately the same proportions of the $C_{16}$ aldehydes as the helicoverpids but has incorporated (Z)-9-tetradecenal, tetradecanal, and (Z)-11-hexadecenol into its pheromone bouquet (Fig. 31). Of these additional compounds, (Z)-9-tetradecenal has been shown to stop the attraction of H. zea males (Mitchell et al., 1976).

The premating reproductive isolation of H. virescens from its close relative, H. subflexa, seems to parallel the H. armigera-H. punctigera case in Australia. Postmating reproductive isolation is incomplete between the former species pair and the development of premating isolation should be promoted rapidly through strong sexual selection. Heliothis subflexa has a more restricted host range and geographical distribution than H. virescens and would be expected to respond most rapidly to this selection pressure. This response is indicated by the pheromone blend. Not only has this species incorporated mono-unsaturated $C_{16}$ acetates but it also has developed a ratio of the $C_{16}$ aldehydes that is altered drastically over the primitive condition (Fig. 31).

Although both blend ratio differences and the presence of the acetates in the H. subflexa pheromone probably maintain species specificity, only the acetates have been shown to be of importance for the attraction of H. subflexa males and the inhibition of H. zea male attraction. Nonetheless, the altered ratio of the $C_{16}$ aldehydes may function in close-range courtship (Teal et al., unpublished).

The incorporation of $C_{14}$ aldehydes by H. virescens and of $C_{16}$ acetates by H. subflexa was probably accomplished quite easily biochemically and would have responded to selection rapidly in order to promote the isolation of these species from each other and from H. zea. However, no data are available on the volatile components of the pheromone released by calling females nor have extensive studies been conducted to determine the roles of individual components in reproductive isolation. Until such studies are completed, the details of pheromonally induced reproductive isolations will remain unknown. Such knowledge may, however, be important to the implementation and sustained use of large-scale control programs. The evolutionary considerations outlined above indicate both the importance of the pheromone blend to reproductive isolation and the rapid response of the blend to selection pressure.

Figure 31. Chemicals identified from pheromone gland extracts of H. zea, H. virescens and H. subflexa. Based on data of Klun et al. (1980a,b) and Chapter VI. For ease of comparison, (Z)-11-hexadecenal equals 100 for all species and other compounds are graphed as percentages of (Z)-11-hexadecenal. Chemicals considered: 1 = tetradecanal, 2 = (Z)-9-tetradecenal, 3 = hexadecanal, 4 = (Z)-7-hexadecenal, 5 = (Z)-9-hexadecenal, 6 = (Z)-11-hexadecenal, 7 = (Z)-7-hexadecen-1-ol acetate, 8 = (Z)-9-hexadecen-1-ol acetate, 9 = (Z)-11-hexadecen-1-ol acetate, 10 = (Z)-9-hexadecenol, 11 = (Z)-11-hexadecenol.


Chemicals Identified
Relative Concentration
100
75
50
25
100
75
50
25
100
75
50
25
1 2 3 4 5 6 7 8 9 10 11

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